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CHANGES OF CERTAIN CHARACTERS OF THE BLOOD OF DAIRY CALVES DURING ONE YEAR OF LIFE AND THEIR POSSIBLE RELATION TO RATE OF GAIN IN BODY WEIGHT[1]

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(Received for publication May 28, 1955)

Several investigators (3, 4, 5, and others) have expressed the opinion that each individual of a species probably has a blood picture peculiar to itself, subject to variation as a result of environmental conditions. The theory would appear to be logical, since each individual animal has its own set of homeostatic mechanisms that maintain the equilibrium of certain blood constituents. These mechanisms probably are of variable efficiency in different individuals and should result in a blood picture that is peculiar to the individual. If such an individual blood picture does exist, there is the possibility that it may be indicative of the growth, lactational, and/or other physiological abilities of the individual.

The study reported in this paper was made to obtain information on six blood constituents in healthy dairy calves from birth to 1 year of age. The relation of gain in body weight to the blood character values was also studied.

METHODS

Twenty-nine purebred Holstein heifer calves in the University herd were used in this study. Blood was obtained from the jugular vein at approximately 3 week intervals (more frequently in many cases). The blood determinations were begun shortly after birth in most calves, begun about the 40th day of life in others, and continued through 1 year of life or longer. Coagulation was prevented by adding 20 mg. sodium oxalate to 10 ml. of whole blood. All determinations were made within a few hours after collection, with the excep-

[1] Published with the approval of the Director as Paper No. 683, Journal Series, Nebraska Agricultural Experiment Station.

tion of the eosinophil count, it being made the day after collection. Collections were made between 8:30 and 9:30 A.M. to avoid possible diurnal variation.

The blood characters evaluated and the method of evaluation are the following: Erythrocyte count was determined in a Spencer bright line counting chamber. Sulfhydryl content was determined by the method of Flesch and Kun (6) and is expressed as milligram per cent with glutathione as a standard. The values probably are not total sulfhydryl content since no denaturing agent was used and are of comparative value only. A phosphate buffer (pH 7.2) served as the buffering agent for the blood. Glucose levels were evaluated by the method of Somogyi (13) and Nelson (10). Hemoglobin content as oxyhemoglobin was colorimetrically determined in a weak ammonium hydroxide solution (8). Eosinophil count was made according to the method of Pilot (11). Hematocrit value was determined by per cent cell volume after centrifugation at 2200 r.p.m. for 15 minutes in a Wintrobe tube.

The functional importance of red blood cell count, blood glucose levels, hematocrit values, and hemoglobin content are explained in standard physiology texts and need not be discussed here. The physiological significance of sulfhydryls is discussed in detail by Barron (1) and the possible relation to mature size in rabbits in Gregory and Goss (7). The significance of eosinophil count and its relation to adrenal cortical function and homeostasis is discussed by Sayers (12). It was because of the indicated physiological significance of each of these characters that they were selected for study. Many others might as justifiably been chosen, but because of limited help and facilities these six were studied.

An analysis of variance of the data on the six blood character values was made, classifying the data according to age and individual calf. Thus the statistical significance of the differences of the means between calves and between monthly age periods was determined.

Body weights were taken at each blood collection time. These individual weights were plotted against age on arithmetic graph paper. An average line was drawn through the plotted weights points. From this line the rate of gain in body weight was evaluated for a particular age. The average rate of gain was estimated for a 10-day period, the period centered on the days of age recorded in Table 1. Thus the

self-accelerating growth phase could be differentiated from the self-inhibiting growth phase (2). Correlation between each of the six blood character values and concurrent short-time rate of gain was made. Also the means of each blood character for the individual calf during a specific age period were correlated with the individual's rate of gain from 100 to 360 days of age. Thus the degree of association between blood values and concurrent rate of gain and the between calf values and long time gain during the first year of life was determined.

Results

Considerable variation occurred in each of the six blood characters between calves and within the same individual at different times. Calves whose blood tended to be low or high in certain values in comparison to similar blood values in other calves seemed to maintain the individual relationship from one time of blood sampling to the next. An analysis of variance of the values was run to determine whether there were significant differences between calves in certain of the blood character values. Since age seemed to be a definite factor in influencing the values, its influence was determined in the analysis. Table 1 summarizes the variance existing in the data obtained with the blood of the 29 calves studied.

There is a highly significant difference between age groups (30-day period) for the six characters determined. Also there is a highly significant difference between the means for individual calves, when the variance due to age is removed, for erythrocyte count, sulfhydryl content, hemoglobin level and hematocrit value. Kunkel et al. (9) have established the individuality of blood glutathione levels for beef calves. There was no significant difference between calves for glucose level and a low significance for eosinophil count. These data indicate that each calf has its own peculiar blood level in erythrocyte count, sulfhydryl content, hemoglobin content, and hematocrit values. Factors other than age, undoubtedly, influence the level of these characters—such a factor as season of the year. An attempt was made to evaluate the effect of season in these data but because 22 of the 29 calves were born during winter and spring months and only 7 in summer and fall months accurate determination of seasonal effect was not considered possible. Nevertheless there is a strong indication that several of the six blood character values are affected ma-

TABLE 1
Analysis of Variance of Six Blood Character Values Classified for Age and Individual Calf.

Source of Variation	Degrees of freedom	Mean Squares of Blood Values					
		R B C millions	–SH mg. %	Glucose mg. %	Hemoglobin grms/100 cc	Hematocrit Vol. %	Eosinophils numbers
Age (30 day periods)	11	15.49**	2583**	547**	2.28**	84.84**	63900**
Calf	28	3.97**	571**	87	2.62**	56.51**	30300*
Error	308	1.25	132	85	0.95	12.18	17500

** = Significant at 1% level.
* = Significant at 5% level.

TABLE 2
Blood Character Values and Gain in Body Weight.

No. det.	Age group (days)	Mean age (days)	Mean body wt. (lb.)	RBC (1,000's per mm.)	–SH (mg/%)	Glucose (mg/%)	Hemoglobin (gp/100ml.)	Hematocrit (vol. %)	Eosinophils (no/mm.^{3})	Mean gain (lb/day)
20	0- 30	15	92	8,501	187	103	9.8	34.7	112	.80
34	30- 60	48	131	9,759	160	75	10.2	37.5	129	1.35
36	60- 90	77	183	10,112	150	71	11.1	40.1	114	1.75
60	90-120	105	237	10,262	147	70	11.1	39.7	122	2.00
45	120-150	136	294	10,046	144	70	11.3	39.0	186	2.00
37	150-180	165	350	9,411	140	67	11.0	39.2	169	2.00
54	180-210	194	408	9,347	136	63	10.5	37.1	147	1.80
44	210-240	223	454	9,233	136	63	10.5	36.0	185	1.70
40	240-270	252	500	9,278	137	63	10.6	35.2	164	1.60
42	270-300	282	542	8,830	138	64	10.9	35.4	170	1.50
38	300-330	314	589	8,527	139	61	10.8	35.7	307	1.50
38	330-360	348	638	8,388	138	61	10.8	36.2	240	1.55
32	360-390	375	680	8,494	140	63	11.1	36.7	260	1.60

terially by season. This is especially true for blood glucose level. Future work will be directed toward a more accurate evaluation of seasonal influence.

The changes in values of the six blood characters with increasing age during the first year of life for all calves are shown in Table 2. The mean rate of gain for all calves at monthly intervals is shown.

Erythrocyte count reached a peak value at about the fourth month of life then decreased gradually up to a year. The coefficient of variation exclusive of that due to age and individuality of the calf was about 12 per cent of the mean value of 9.3 million erythrocytes per mm^3.

Sulfhydryl value was high at, and shortly after, birth and gradually declined to the time when the peak in rate of gain was attained. From then to one year the value appeared to change little with age. The coefficient of variation for sulfhydryl value was about 8 per cent of a mean yearly value of 140 mg per cent after the effect of age and calf was removed.

Blood glucose was high during early life and decreased gradually with increasing age. The coefficient of variation again excluding variation due to age and calf individuality was about 14 per cent. The mean yearly glucose level was 66 mg per cent.

Hemoglobin values change little during the first year of life. Low values during the first month of life were usually associated with low erythrocyte count at this time. The coefficient of variation was about 9 per cent. The mean yearly value was 10.8 gms/100 ml.

Hematocrit values, in general, rose to the third month of life, tended to remain high for several months until the rate of gain declined materially, then declined and remained at a constant level during the last 5 or 6 months of the first year of life. The coefficient of variation was about 9 per cent. The mean hematocrit value for the year was 37.3 per cent.

The eosinophil counts during the first year of life in the study were low with wide variability. The means are not very informative because of this variability; but a tendency for the number to increase with age was indicated.

Data present in Table 2 shows that on an average the peak in rate of gain was attained about the sixth month of life. For individual calves the peak was attained at different ages. Season of birth ap-

parently affected the time at which greatest gain was attained in these calves. Rate of gain was markedly reduced during the hot summer months especially in those calves over 3 or 4 months of age and tended to increase again about September. Thus the peak in rate of gain was delayed until fall in calves that reached 6 months of age during the summer. In somewhat older calves, the peak rate of gain was attained before the summer months, and although the gain again increased in the fall, it did not reach the rate attained in the late spring.

Individual values for the six blood characters were correlated with the rate of gain during a period ± 5 days of the time the blood determinations were made. The correlation coefficients obtained with individual values and this short time current rate of gain were as follows:

Erythrocyte count vs current 10 day gain = +0.173**
Sulfhydryl value vs current 10 day gain = +0.058
Glucose value vs current 10 day gain = +0.185*
Hemoglobin value vs current 10 day gain = +0.090
Hematocrit value vs current 10 day gain = +0.253**
Eosinophil number vs current 10 day gain = +0.096

Erythrocyte counts, glucose levels and hematocrit values were significantly associated with concurrent short time rate of gain (409 degrees of freedom) but the relationship is of a low order. Closer inspection of these relationships for individual calves show that peak in erythrocyte count is reached before peak in rate of gain and declines before rate of gain declines, thus contributing to a low order of relationship. The positive correlation with glucose appears to be largely due to the fact that glucose decreases in the summer and increases in the cooler months similar to the seasonal fluctuation in rate of gain.

Hematocrit values seem to be more closely related to concurrent gains than any other factor.

Sulfhydryl is negatively related to rate of gain during the first six months of life, but there is a strong tendency for this value to fluctuate with season, just as rate of gain does. Thus, there is a low non-significant over-all relationship.

Hemoglobin values are relatively constant and do not markedly change with concurrent rate of gain.

Eosinophil count varies considerably from one time to the next and shows little or no relationship with concurrent gain.

Contributing also to all these relationships is the fact that estimation of the rate of gain over a 10 day period is probably inaccurate for an individual case and even though a relationship might exist between any of the blood characters and concurrent gain, it would probably not be very high due to the inaccuracies in estimating gain.

Correlation between the average value of each of the six blood characters obtained when the calves were 90-125 days of age and the gain for individual calves from 100 to 360 days of age was made. The differences between calves were greater, and within calves, more constant from 90-125 days of age than at earlier or later ages. This is thought to be because recovery from birth had taken place by this age and all calves were on a more uniform basis of handling and feeding at this period than at a later time. Relationships between grouped values for individual calves at the 90-125 day period (from 3 to 6 values per calf) and rate of gain in body weight from 100-360 days of age are shown in the following (27 degrees of freedom):

Erythrocyte count vs 100-360 day gain	$r = -.32$
Sulfhydryl value vs 100-360 day gain	$r = -.51^{**}$
Glucose level vs 100-360 day gain	$r = -.05$
Hemoglobin content vs 100-360 day gain	$r = -.35$
Hematocrit value vs 100-360 day gain	$r = -.38^{*}$
Eosinophil count vs 100-360 day gain	$r = -.13$

Sulfhydryl and hematocrit values were significantly related to gain for a period of 260 days following the time the blood determinations were taken at the 90-125th day of life. The relationship was negative for gain during the period from 100-360 days compared to the low positive relationship existing for concurrent short time gain. This may indicate that calves gaining faster at an early age tend to gain more slowly during the later part of their first year of life. Thus individual body weights at 100 days of age might be expected to be related negatively to gains from 100 to 360 days. However, such a relationship was found to be low and non-significant, possibly because a calf's body weight at 100 days did not necessarily represent its growth rate in early life. At any rate opposite relationships occurred between blood factors and concurrent gain and between blood

factors at 90-125 days of age and future long time gains (100-360 days of age).

A multiple correlation using the two factors, sulfhydryl content and hematocrit values, against gain during the 100 to 360 day period gave an R value of 0.53. The relationship between sulfhydryl and hematocrit was $r = +.47$. The multiple correlation is probably not significantly higher than the single correlation for sulfhydryl alone for this set of data; but when values for hematocrit and sulfhydryl at age periods other than the 90-125 day period against future gains were correlated, the multiple correlation using both sulfhydryl and hematocrit was consistently more highly related to future gain than either one alone. The ratio between these two blood factors seemed to be important in predicting gain over the 100-360 day period. It appeared that the balance between characters was probably as important as the actual values. Multiple correlations with rate of gain from 100 to 360 days of age were made with erythrocyte count and sulfhydryl values, erthyrocyte count and hematocrit values, and hemoglobin content and sulfhydryl values. None of these multiple relationships showed a higher value than that obtained with hematocrit and sulfhydryl values. However, erythrocyte count and hematocrit and erythrocyte count and sulfhydryl showed a relationship to the 100-360 day gain almost as high as that of the hematocrit and sulfhydryl correlation. Additional data on more calves is essential to justify confidence in the above discussed relationships.

Since season probably affects values of these blood characters, proper evaluation of the influence of season should be made. Individual calf values for a specific age might then be more properly placed on a comparable basis when correlated with future gain. Other factors, no doubt, such as ration influence values and work should be carried out to evaluate these if possible. Refinement in evaluating blood characters may lead to valuable information concerning the physiology of dairy cattle as well as other animals.

Summary

Periodic evaluation of six blood characters from birth to approximately one year of age for 29 Holstein calves were made. They were erythrocyte count, sulfhydryl content, glucose level, hemoglobin value, hematocrit value and eosinophil count. Significant changes

occurred in the values of each of these characters with advancing age during the first year of life. Significant differences between calves for erythrocyte count, sulfhydryl content, hemoglobin content and hematocrit values were found if variance due to age was removed. Hematocrit values were more closely related to concurrent rate of gain than any of the other characters. Sulfhydryl and hematocrit values of the blood of individual calves 90-125 days of age appeared to be significantly correlated with their rate of gain from 100 to 360 days of age.

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THE GROWTH RESPONSE OF CORN SEEDLINGS TO NORMAL AND CANCER RAT BLOOD*

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(Received for publication June 14, 1955)

INTRODUCTION

The results of studies concerned with the effects of chemical regulators on plant growth have become so firmly established as to find a tremendous application in the agricultural field. However, the effects on plant growth resulting from the introduction to the growing media of biologicals of animal origin appear not to have received consistent study except from the "phytopharmacological" school of Macht. This investigator has an extensive series of publications, spanning more than thirty years (Macht, 1922, 1954), dealing with the effects of human blood sera, including that of individuals with varying diseases, on the root growth of Lupinus alba. Seedlings were oriented so their roots grew downward into the test solution, containing a diluted serum sample, and after a suitable growth period root lengths were compared with seedlings grown in a nutrient (Shive's) control solution. The differences in root length gave a "phyto-toxic" index which was found to be quite characteristic for certain diseases (Macht, 1949, 1954).

In recent years, one of us (J.M.W. 1951) investigated the corn seedling as an appropriate phytobiological test agent and employed a factorial design to determine the effects on the growth of test plants of a number of variables such as period of germination, temperature, concentration and pH of media. The effect of variations in the primary root length on the subsequent growth of seedlings was also determined. This earlier study established the range of controls necessary to the growth environment which would assure a consist-

* This project has been supported by a grant from the South Dakota division of the American Cancer Society.

ent growth pattern on repeated determinations and would yield results of some statistical value. In the course of this experiment it was noted that growth of the seedling plants was not suppressed to the same degree when blood from cancer patients supplanted normal blood in the growing media. Whether or not this response was due to alterations in the blood resulting from the cancerous condition was difficult to state since the blood donors were from a widely variable group both as regards physical condition, type of therapy in progress, and types of tumors represented.

In view of these variables it appeared expedient to determine the efficacy of the corn seedling as a phytobiological test agent under the optimally controlled conditions provided by the animal experimentation described in this report.

Method

Two cancer strains were used, namely the Walker carcinosarcoma 256 and the Jensen sarcoma.* Although equal numbers of the two strains were used, our results fail to define any differential effects peculiar to either tumor. Thin slices of tumor taken from the host growth 10 to 14 days of age were transplanted into the axilla of mature Sprague-Dawley rats without discrimination as to sex. There did exist considerable variation in early growth, it varying from 3 to 10 days for the transplant to attain 2 to 3 cm. in size. This variation in age prevented an original attempt to analyze data on the basis of age of the tumor, or days following transplantation, and hence the results are tabulated on a basis of tumor size with only incidental reference to age.

Heparinized blood samples were collected by direct cardiac puncture under sterile conditions with the animals maintained under light ether anesthesia. Usually the animal was exsanguinated at the time but in many instances only minimal amounts of blood were taken and the animals were maintained varying periods of time for repeat determinations as the tumors attained an increased size (Table 2). Blood collections from normal animals of like age and strain were made in a similar fashion, but in this instance the animal was used for only the one determination.

* The original stock was kindly provided by Dr. K. Sugiura of the Sloan-Kettering Institute for Cancer Research.

The corn seeds were selected for uniform type (Pioneer Hi-Bred Corn Company, number X0900) and size, sterilized in bromine water for one hour, repeatedly washed in distilled water, and germinated for 48 hours at 25° C (Figure 5). Five selected seedlings having primary roots of 1 cm. in length were placed on filter paper in petri dishes (Figure 6). The following solutions were then added to a group of three such dishes, or a total of fifteen seedlings for each solution. One group of seedlings was grown in distilled water to serve as a check or control to determine that growth conditions were genuinely uniform (Figure 6). Ten cc. of an 8% solution of blood from the normal and cancer rats were respectively added to each of the three dishes comprising the second and third groups of seedlings (Figure 6). The petri dishes were covered and incubated at 28° C for 72 hours (Figure 7). The entire root systems were then cut from the seedlings, dried at 103° C for 24 hours and weighed to the nearest 0.2 mg. (Figure 8). The individual weights for seedlings in the given solutions were graphed (Figures 1, 2), and the average differences in dry weight for the 15 seedlings grown in cancer blood were compared with the root weights for those grown in normal blood solutions (Figures 3, 4).*

Observations

The results to be reported were obtained from 53 separate determinations (Figures 3, 4) representing a total of 42 cancer animals. A comparison of typical growth curves (Figures 1, 2) serves to illustrate the general type of variation to be expected, both among seedlings of a single determination as well as the variation in average weights for successive determinations. As a general rule a lower average root weight for seedlings grown in blood solutions was reflected in a lower average weight in the water grown controls, suggesting that slight deviations of temperature in the incubator do result in a significant variation in growth weights. Indeed the water controls serve as a check on the constancy of the growth environment. The variations in root weights of seedlings in any given run (Figures 1, 2) whether grown in water or blood solutions may indicate the existence of genetical factors in growth which cannot be

* The authors acknowledge the assistance of Clara K. Winter in handling the test material.

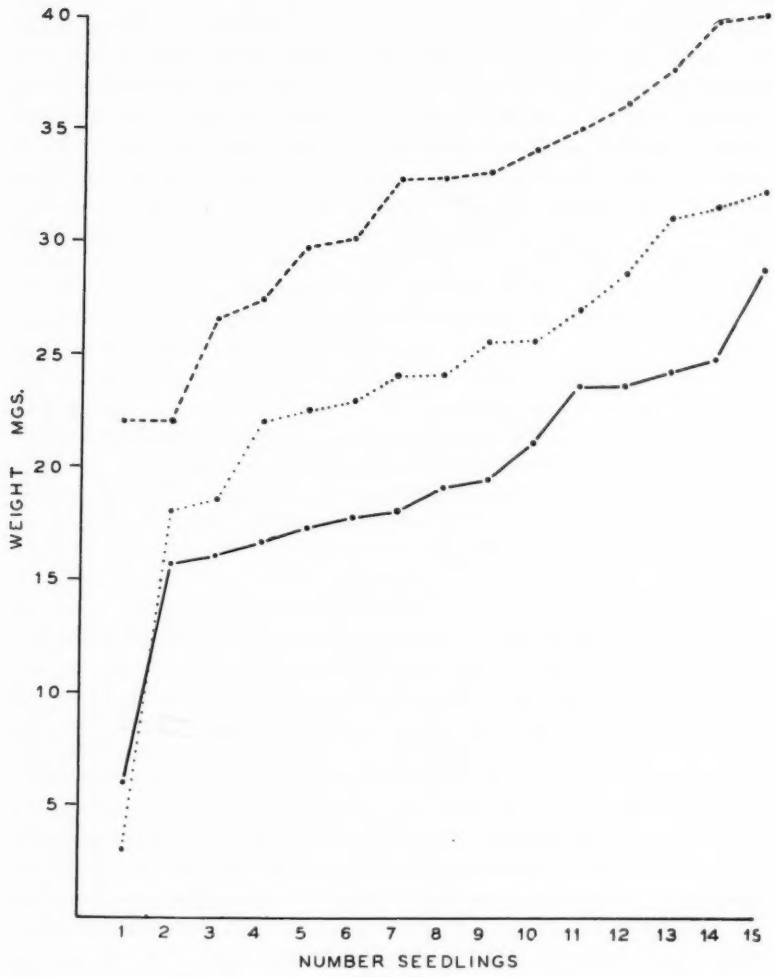

FIGURE 1

Root weights for individual corn seedlings grown in water (- - - -), and in blood solutions from normal (———), and cancerous (. . . .) rats.

entirely compensated for despite a careful selection of kernel size and standardization of the seedling by using only those with a uniform length of primary root (Figure 6).

The degree of depression on growth exerted by blood solutions of a normal animal as compared with a water grown control is illustrated in Figures 1 and 2. Reactive blood solutions from cancer

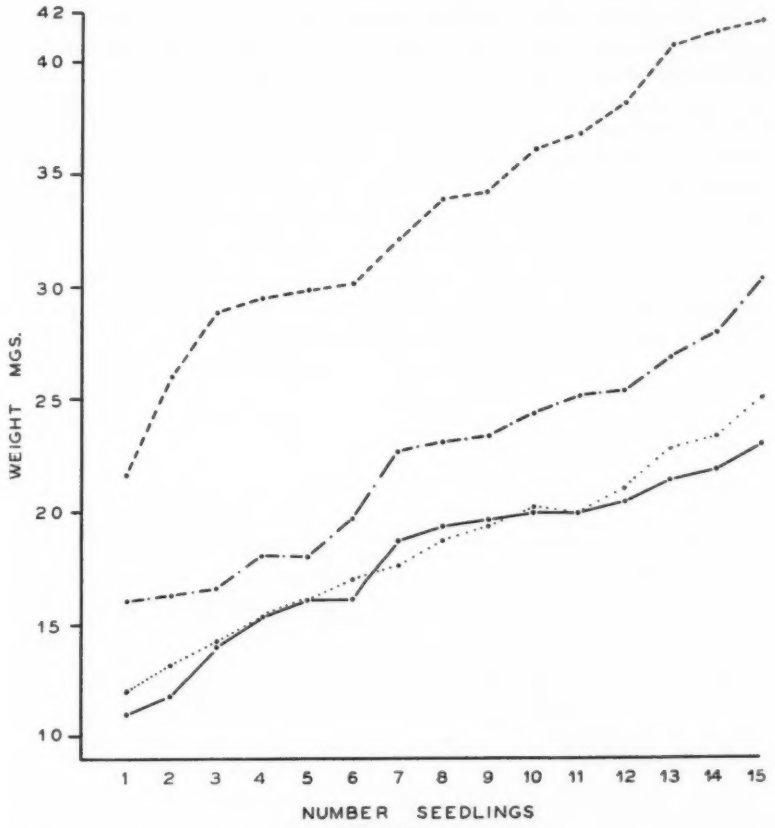

FIGURE 2

Dry root weights for corn seedlings grown in blood solutions from two cancer rats, numbers 15 (....), and 16 (— - —), compared with blood of a normal rat, number 17 (———), and the water grown control (- - - -).

animals do not exert the same degree of depression as measured by dry root weights. Figure 1 represents a typical response for, in this instance, an animal with a large tumor 6 cm. in size and 18 days following transplantation. The average root weight increase for this animal blood was 4.37 mg. This same animal (Figure 2, no. 15) had been bled 9 days following transplantation, but the blood exhibited virtually no effect on growth as compared with the control specimen. Blood from another cancer animal (no. 16) was evaluated in the same trial with number 15 (Figure 2) and did exhibit an average root weight increase of 4.40 mg. as compared with control animal number 17. A study of the two sets of data, that is, root weights grown in cancer blood as compared with normal blood, revealed a high degree of correlation, $r = .82$.* Consequently the results were analyzed on the basis of paired variates using the familiar "t" test to determine whether or not the differences were statistically significant. It has seemed expedient, for reasons cited, to analyze the data on the basis of size of cancer rather than days following transplantation from the donor tumor. Tumors were classified as small (2-3 cm.), medium (3-5 cm.), and large.

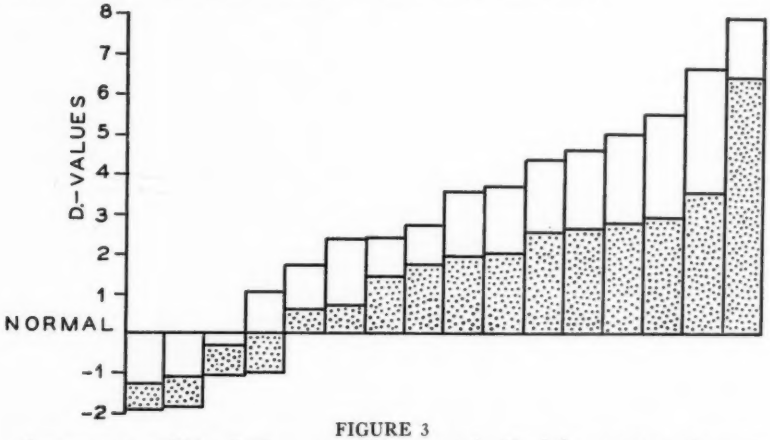

FIGURE 3

Average root weights of 15 corn seedlings grown in blood from cancer rats compared with the normal controls. Stippled frames represent animals with small tumors; open frames those with large tumors.

* The authors are indebted to Dr. W. W. Gutzman, Professor of Mathematics, University of South Dakota, for his statistical analysis of the data.

Sixteen determinations were made on animals bearing small tumors (Figure 3). These tumors varied in age from 3 to 13 days with 10 of the 16 individuals confined to a 5 to 9 day interval. In four cases the root weights were lower than those of seedlings grown in normal blood while the remainder exhibited increased weights varying from 0.59 to 6.39 mg. and an over-all average increase of 1.42 mg. Statistical analysis gave a $t = 2.64$ which is significant at the .05 level. Repeat determinations were made at a later date on two (numbers 22, 42) of the four cases yielding negative values and in both instances (22a, 42a) marked increases in root weights over control bloods were obtained (Table 2).

The most sharply defined results were obtained from animals bearing tumors of from 2 to 5 cm. in diameter, with the majority varying in age from 10 to 15 days. In a total of 21 separate determinations (Figure 4) involving a like number of animals, root weight

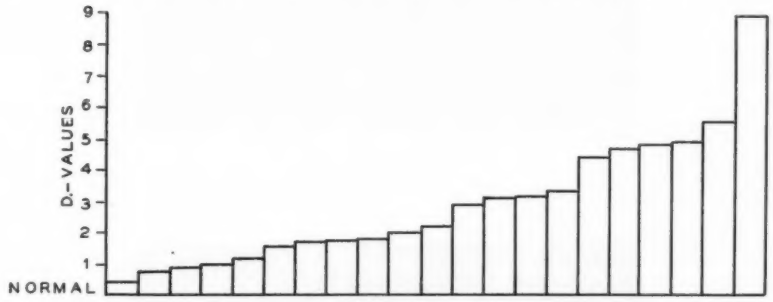

FIGURE 4

Average increase in root weights, compared with normal controls, for seedlings grown in 21 samples of cancer blood from rats with tumors of medium size.

differences between cancer blood and the normal controls varied from a minimal increase of 0.40 mg. to a maximum of 8.90 mg. with an average difference of 2.91 mg. Statistically an analysis of the paired variates in the 21 cases yielded a "t" value of 6.8 which is significant at the .001 level.

Data obtained from animals with small and medium sized tumors were purposely segregated from the large cancer group because of the extreme dissimilarity in the physical condition of the two groups of animals. Those with smaller tumors appear robust, eat well, and

their physical condition appears good. On the contrary, the animals bearing large tumors are in a debilitated state, emaciated, and it is quite apparent their metabolism is markedly deranged. Since this lowered general physical condition could interject many unknown factors which potentially at least could affect the growth response as herein encountered, it appeared logical to analyze data from such animals distinct from the comparatively healthy group.

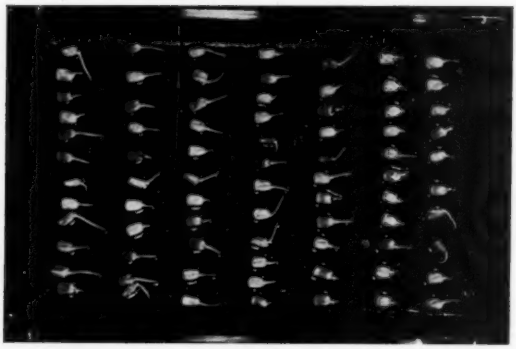

FIGURE 5
Corn seedlings following a 24 hour germination period from which are selected test seedlings for uniformity of primary root length.

FIGURE 6
Selected seedlings, preparatory to incubation, in blood solutions from normal and cancer animals and a water control.

Bloods were analyzed from a total of 16 rats (Figure 3) bearing large tumors varying in ages following transplantation of from 16 to 31 days. In three instances average weights of seedlings grown in cancer blood were less than those for the normal control. For the remainder, increased root weights for the cancer blood varied from 1.1 mg. to 7.92 mg. The entire series showed an increase of 3.05 mg. in root weights for seedlings grown in cancer blood as compared with

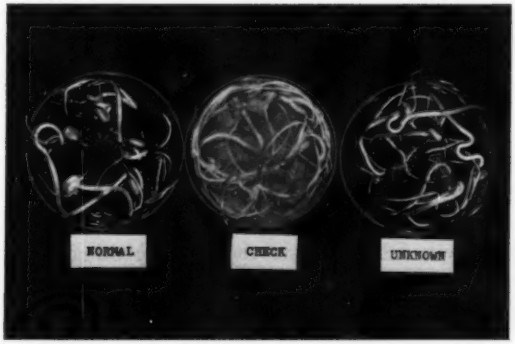

FIGURE 7
Seedling growth following a 72 hour incubation period.

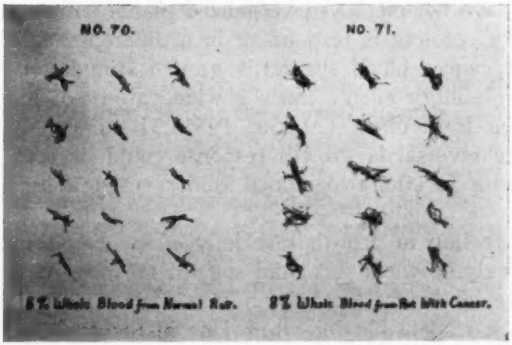

FIGURE 8
The appearance of dried roots from fifteen seedlings grown in blood solutions from cancer bearing and normal rats.

the normal controls. An analysis of the data yielded a "t" value of 4.62, significant at the .001 level, or essentially the same level of significance for the bloods obtained from animals having medium sized tumors.

Discussion

The factors in blood of cancer animals which are responsible for promoting an increased growth rate are obscure at the present time. Peculiarly enough, the responsible agent(s) appears not to be a function of the blood serum alone. At least, determinations using only the serum fraction of the cancer blood failed to yield any consistent differences in root weights when compared with normal blood sera. It must be noted, however, that the rate of growth for seedlings in blood sera is very close to the more optimum growth environment provided in the water grown controls, and hence any differences which might occur are of a sufficiently low order as to go undetected.

The fact that the use of blood sera does not result in a significant deviation in growth rate suggests the corn seedling is responding in a somewhat different manner than the plant Lupinus alba employed by Macht (1954). This author reports a suppression of growth, or phyto-toxic effect, amounting to 20-30% for normal blood serum, but this inhibition is markedly increased for human sera embracing a wide variety of cancers. It is to be noted that the toxic effect was increased on vernalized plants in contrast with normal plants. Although we have not employed vernalized plants it would still appear that the corn seedling is responding in a different manner since our results with cancer blood suggest a growth stimulation rather than a toxic or inhibitory effect. Since a wide variety of human cancers exhibited the toxic effect (Macht, 1951), it appears unlikely that the complete reversal in growth response could be attributed solely to peculiarities for the two animal tumors used in the present experiment.

The suppression in growth rate induced by blood solutions from normal animals (Figures 1, 2) did suggest the possibility that hemoglobin is the inhibitory factor, and that variations in its concentration could be responsible for alterations in the growth response. If such were the case, then the degree of inhibition would be identical for either normal or cancer blood. Conversely, lower concentrations of hemoglobin should result in higher growth rates assuming

hemoglobin concentration to be the only factor modifying growth under these conditions. A number of observations mitigate against such an interpretation and suggest that other factors than hemoglobin alone are responsible for alterations in the growth weights.

Blood hemoglobin concentrations were determined for a total of 29 of the tumor bearing animals, and for the normal controls, at the

TABLE 1
Differences in Root Weights for Cancer Animals When the Hemoglobin Concentration Was Within Normal Limits, on Left, or Reduced.

Animal	Tumor Size	Hemoglobin % Normal	D Value	Animal	Tumor Size	Hemoglobin % Normal	D Value
21	medium	+ 2	4.99	18	small	35	4.59
31	small	12	3.49	32a	large	28	2.67
32	small	3	6.39	38	large	40	2.41
35	medium	+ 4	3.14	39	large	30	—1.24
37	medium	0	4.82	42	small	20	—1.85
69	medium	14	2.90	50	medium	30	.85
79	small	+ 2	1.40	52	medium	42	2.00
81	medium	+ 6	1.20	54	medium	40	1.60
84	small	15	2.50	48	small	26	2.58
87	small	7	2.70	50a	large	42	1.10
				61	medium	33	1.00
		Average	3.35	63	medium	25	1.80
				67	large	60	5.00
				71	medium	42	8.90
				73	large	48	2.40
				46a	medium	27	0.40
				83	medium	36	2.20
				86	medium	64	0.80
				84a	large	32	6.60
						Average	2.30

time blood samples were collected (Table 1). Since there was as much as 15% variation among the normal animals, the tumor animals were classified as having hemoglobin concentrations either within normal limits or in a reduced amount. Blood from animals having hemoglobin concentrations within normal limits resulted in an average root weight increase of 3.35 mg. compared with the normal bloods, while those animals exhibiting markedly reduced hemoglobins showed an average root weight increase of only 2.35 mg. Further, were differences in growth weights the result of variations in hemoglobin concentrations alone, one would expect a consistent root weight increase for bloods in which the hemoglobin concentrations were uniformly low, as for animals with advanced cancer, as

compared with the high hemoglobin levels. This hypothesis is not confirmed by the determinations (Table 1). Cancer animals with hemoglobin concentrations in the normal range (ex. 21, 32, 35, 37) yielded an increase in root weights considerably above bloods having only one-third to one-half the amount of hemoglobin (ex. 39, 50, 54, 50a, and 86).

TABLE 2

Increased Root Weights for Two Separate Blood Samples from the Same Animal at Varying Time Intervals Following Tumor Transplantation.

Animal	Tumor Days	Tumor Size	Increase Root Wts., Mg.	
9	5	small	1.95	
9a	18	medium		5.51
10	5	small	1.83	
10a	16	medium		3.12
13	7	small	2.84	
13a	16	medium		4.72
15	9	small	.65	
15a	18	large		4.37
18	11	small	.59	
18a	30	large		4.59
16	9	medium	4.40	
16a	23	large		3.65
22	13	small	—1.07	
22a	23	large		3.51
32	9	small	6.39	
32a	18	large		2.67
50	10	medium	.85	
50a	19	large		1.10
84	11	small	2.50	
84a	16	large		6.60
42	3	small	1.85	
42a	10	medium		1.75
		Average	2.07	3.78

It is interesting to observe the increased root weight difference in successive blood samples from the same animal but with an increase in tumor size, or age following transplantation. A total of eleven rats were bled in a period of from 3 to 11 days following trans-

plantation (Table 2). Four of these bloods failed to show significant effects on root weights. The average increase in roots weights proved to be 2.07 mg. These animals were bled a second time in periods ranging from 7 to 19 days following the first bleeding. In all but three instances an absolute increase in root weight occurred. In two of the three cases where a decrease in root weight from the previous trial was obtained the difference was of a very low order. The average increase in root weight for the entire group proved to be 3.78 mg. as compared with an average of 2.07 mg. on the first determinations. These observations suggest that whatever factors may be responsible for the stimulation of growth on root seedlings, the effect is cumulative either with increased tumor size or length of time it is in contact with the host. This observation is also supported by the higher statistical significance for the growth increases observed in animals bearing medium to large tumors in contrast with the younger or smaller tumors.

Theoretically, if there is an increase in some growth stimulating factor induced by the tumor, this effect could be abolished by destruction of the tumor. This hypothesis needs confirmation in a substantial series of cases and is presently being studied, but one case appearing in the present series is worthy of mention. A blood sample was taken from host number 21 which had received a transplant 13 days earlier. The tumor was of medium size, showed no external skin ulceration, but did appear grossly dark in color suggestive of extensive necrosis. The root weight of the seedlings grown in this blood exceeded the normal control by 5.01 mg. Eleven days later, or 24 days following transplant, the tumor had disappeared and in its site was an open crypt-like lesion, with necrotic and granulation tissue in evidence. A blood sample drawn at this time was essentially unresponsive as compared with the normal control, the root weight exceeding the control by only 0.47 mg. Twenty-four days later, or 48 days following transplantation, the animal exhibited a completely healed skin in the site of the original lesion and no evidence of an existing tumor. The root weight of seedlings grown in a blood sample on this date deviated from the normal by an insignificant 0.24 mg.

While the factors responsible for the type of growth response described in this experiment are presently undetected, it seems evi-

dent that this technic employing plant material as the growth responsive agent holds some promise for screening reactive biologicals of animal origin.

Summary

A method is described which permits a sufficient standardization of the conditions for the growth of corn seedlings to assure reproducible results with statistical validity. Under the conditions outlined, eight per cent solutions of whole blood from normal rats have a pronounced inhibition on growth of corn seedlings, as measured by dry root weight, in comparison with water grown controls. Blood from rats bearing transplanted tumors (Walker and Jensen strains) do not exhibit the same degree of inhibition but rather root weights were increased above those for seedlings grown in normal blood in 47 out of 53 determinations. Growth increases were found to have a high level of statistical significance (.001) for tumors of 3 cm. or larger in size. Applying the "t" test to data accumulated for growth increases resulting from blood of rats bearing smaller, and younger, tumors indicated a significance at the .05 level.

The observations are discussed in relation to other reports attributing toxic effects on plants when grown in solutions containing biologicals of animal origin. The factors responsible for an apparent stimulation of growth in corn seedlings induced by the blood from cancer bearing rats remain undetected by the present experiment.

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Growth, 1955, **19**, 165-168.

EFFECT OF DIETHYLSTILBESTROL UPON THE PELVIS OF THE YOUNG MOUSE[1]

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(Received for publication June 17, 1955)

Some years ago, Gardner (1936) described the effects of estrogenic hormones upon the pelves of mice. It was shown that following treatment with estrogens, an interpubic ligament is formed between the innominate bones. A survey of the literature has not disclosed any similar work using synthetic estrogenic hormones. This report concerns an experiment using the synthetic estrogen, diethylstilbestrol, kindly furnished by Dr. D. C. Hines of the Lilly Research Laboratories.

Mice used in this work were from Dr. L. C. Strong's C3H colony. The animals were weaned at 21 days of age and divided into four groups (see Table 1). Group A consisted of male mice which received weekly subcutaneous injections of 240 mgs. of diethylstilbestrol dissolved in ethyl oleate. Group B consisted of control male mice which received the same volume of ethyl oleate each week (.24 c.c.). Group C and Group D contained female mice in the experimental and control groups; they received the same injections as the male mice. Injections were continued in all mice until they were sacrificed. Animals were sacrificed by ether at various ages up to 11 weeks of age. Five mice comprised each age group and 90 mice were studied in all. Toxic effects of the hormone resulted in the death of 19 mice. None of these mice or their cage mates are included in the data reported here. No control mice died during the course of the experiment.

[1] The author gratefully acknowledges the criticism and help given by Dr. Frederick H. Kasten, under whose supervision this work was done. This work was done at the Biological Station of Roswell Park Memorial Institute, Springville, N. Y.

[2] GROWTH is pleased to publish the above paper by Roger E. Ganschow, a high school senior who received honorary mention in a recent Westinghouse Science Talent Contest. His sponsor is Dr. F. H. Kasten of Roswell Park Memorial Institute and the paper has been approved by Dr. L. C. Strong and Dr. T. S. Hauschka. GROWTH believes that the publishing of such manuscripts is an encouragement to interested students.

Skeletons were dissected free from the skin and viscera and were fixed in 95% alcohol for 2-3 days. Dawson's method (3) was used to stain and clear the bone specimens. Better preparations were obtained by preliminary fat extraction with acetone following fixation. The width between the innominate bones was measured using a binocular microscope with a calibrated micrometer disc. Independent measurements were made twice on all specimens. These data agreed very closely.

The results summarized in Table 1 are illustrated in Figure 1. The distance between the innominate bones in control males and females

TABLE 1
Effect of Diethylstilbestrol upon the Pubic Symphyses in Mice.*

Age in weeks†	Exp. Males (A)	Cont. Males†† (B)	Exp. Females (C)	Cont. Females†† (D)
3	——	.15 (5)	——	.12 (5)
5	.78 (5)	.15 (5)	1.16 (5)	.20 (5)
7	1.44 (5)	.15 (5)	2.32 (5)	.24 (5)
9	2.06 (5)	.13 (5)	3.20 (5)	.20 (5)
11	1.50 (5)	.10 (5)	3.63 (5)	.16 (5)

* Data are expressed as average length of interpubic ligaments in millimeters. Numbers in parentheses indicate number of mice in each group.
† Age at sacrifice.
†† Control mice received injections of ethyl oleate.

was uniformly small in all animals. A slightly greater distance between bones was measured in control females compared with control males. All mice injected with diethylstilbestrol showed a reaction at the pubic symphyses resulting in interpubic ligament formation. Females had a somewhat greater response in interpubic ligament formation than males. The ligament showed a progressive increase in size in experimental mice for all age groups studied in both sexes with the exception of the 11 week male group. This group had a reduced ligament size compared with the 9 week male group.

The changes induced by diethylstilbestrol are similar to those described by Gardner (1936) who used the estrogenic hormones, theelin, ketoestrin benzoate, theelol, equilin, and equilin benzoate. The production of interpubic ligaments in estrogen-treated mice is considered to result from resorption of the innominate bones (2, 1). Other changes noted in experimental mice in the present study included temporary depilation at the site of injection, slight loss of weight, and acute toxic effects. According to Shimkin and Grady

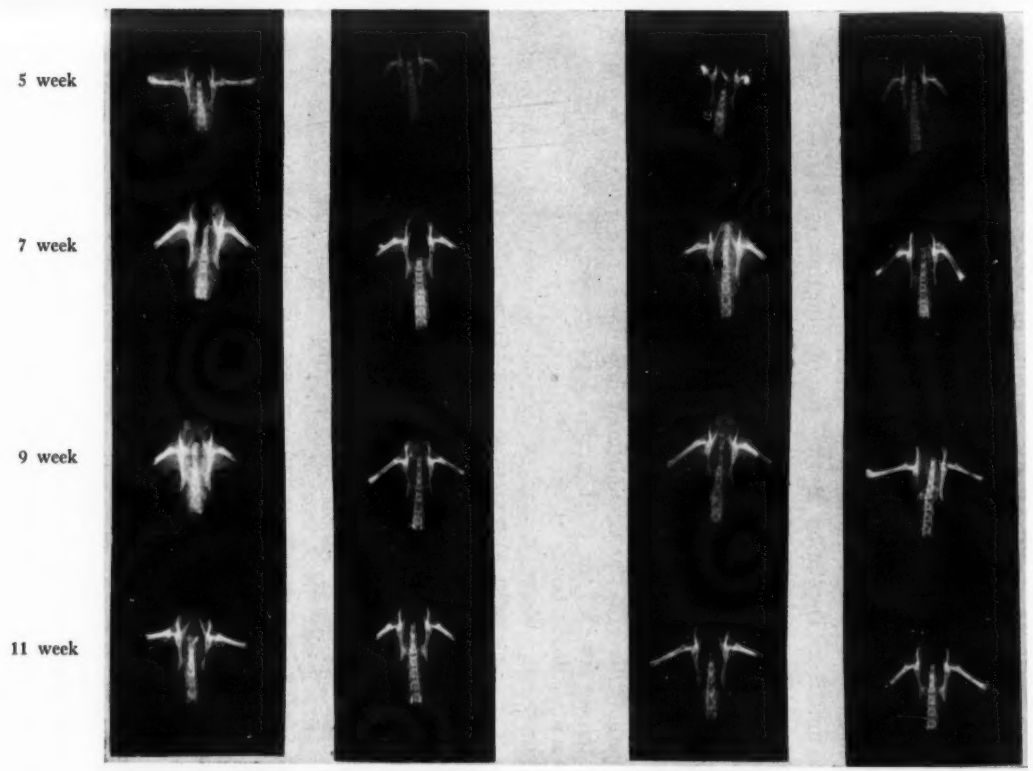

FIGURE 1

An x-ray photograph of samples of pelves from normal mice and mice previously injected with diethylstilbestrol. Specimens were prepared by Dawson's method with alizarine red. Vertebral columns were removed from region of pubic symphyses to allow interpubic distances to be seen in photograph. Note markedly greater interpubic distances in specimens injected with hormone compared with controls.

(1941), the toxicity of diethylstilbestrol to mice is enhanced by the frequency of administration. This relationship was confirmed in the present work. When the hormone was at first administered three times weekly to the entire group of 109 mice, 15 mice died. This schedule was discontinued and the same quantity of hormone was administered once a week. Four more mice died under the revised schedule. Since these latter deaths occurred soon after the change was made, it is probable that toxic effects in these mice had already been initiated. Depilation at the site of injection of hormone was also a feature of the original schedule (three times weekly). The loss of weight observed in mice in the present experiment was mentiond by Shimkin and Grady (1941) in their study of the toxic and carcinogenic effects of stilbestrol.

Summary

Diethylstilbestrol was administered subcutaneously into young male and female mice of the C3H strain. Measurements of the width between innominate bones at the pubic symphyses were made. These measurements showed a marked increase in interpubic distance in both sexes compared with control mice. Other effects noted include depilation at the site of injection, slight loss of weight, and acute toxicity.

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Growth, 1955, **19**, 169-175.

THE EFFECT OF CORTISONE ACETATE ON THE WOUND HEALING PHASE OF *TRITURUS VIRIDESCENS*[1]

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(Received for publication June 24, 1955)

INTRODUCTION

This is the second in a series of papers designed to determine the effects of the cortical steroids on the cells involved in the regeneration of the forelimbs of the adult form of the newt, *Triturus viridescens viridescens* (Rafinesque). The first study (Manner, '53) presented a histological study of regeneration. It also offered a comparison between the mitotic activity of the epidermal and mesenchymal tissues in the regenerating forelimbs.

Three stages of regeneration can be delimited. The first, that of wound healing, begins at the time of amputation and continues for approximately eight days. The second phase, blastema formation, lasts from the eighth to twentieth day after amputation, and the final stage, blastema differentiation, begins on the twentieth day and continues until regeneration of the limb is completed.

Many workers have shown that cortisone exerts an inhibitory effect on wound healing. Baker and Whitaker ('49) painted cortical compounds on the skin of rats and reported a subsequent thinning of the epidermis in the area of application. Ragan et al. ('49, '50) described the suppression of granulation tissue in the healing of wounds inflicted upon rabbits. Creditor et al. ('50) noted that simple incised wounds failed to heal or granulate in patients who were receiving therapeutic doses of ACTH. Baker and Whitaker ('50) applied adrenocortical steroids daily to cutaneous wounds in rats and reported that there was some delay in the closure of the wounds.

Wound healing involves both epidermal and mesenchymal tissue in the forelimb of Triturus. As this animal has large cells and presents a clear picture of wound healing, the following work was undertaken

[1] This investigation was aided by a grant from the National Science Foundation.

as an attempt to localize the action of cortisone acetate in the process of wound healing.

Materials and Methods

The adult form of the newt, *Triturus viridescens viridescens* (Rafinesque), was used in this study. The right forelimb of each of these animals was amputated at a point midway between the elbow and the wrist. Half of these animals were injected daily with .0005 mg. of cortisone acetate. The injections were intramuscular in the caudal region. The rest of the animals served as controls and were injected daily with Holtfreter's solution.

Immediately after amputation and every four days thereafter, eight experimental and eight control animals were sacrificed and their limbs reamputated through the elbow. These regeneration stumps were prepared for histological examination.

In addition to a histological study of the alterations in the wound healing pattern produced by cortisone acetate, mitotic counts of both the epidermal and mesenchymal tissues were made during the wound healing phase. The technique utilized for obtaining the epidermal mitotic index (E.M.I.) and the mesenchymal-epidermal ratio (M.E.R.) is the same as that already described in an earlier paper (Manner, '53).

Experimental Observations

The changes in the epidermal mitotic index and the mesenchymal-epidermal ratio occurring throughout the eight day wound healing period are indicated in Table 1. A graphic representation of the epidermal (figure 1) and the mesenchymal (figure 2) changes are also given.

Immediately after amputation (normal)

The epidermal mitotic index is 0.23%, that of the mesenchymal

TABLE 1

Comparison of changes in the mitotic activity of the epidermal and mesenchymal tissues between animals treated with 0.0005 mg. cortisone acetate daily and control animals.

Days of Regeneration	Epidermal Mitotic Index (%)		Mesenchymal Epidermal Ratio (%)	
	Control	Cortisone	Control	Cortisone
0	0.23	0.23	0.00	0.00
4	0.41	0.17	0.04	0.01
8	0.34	0.62	0.07	0.13

tissue is 0.00%. The epidermis is typical stratified squamous epithelium, the base cells resting on a distinct basement membrane and varying in height from cuboidal to low columnar. The average thickness of the epidermis is three cells.

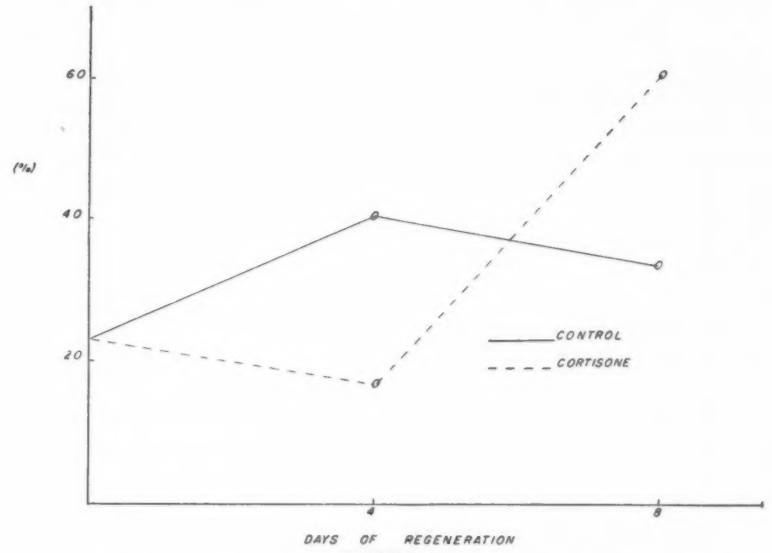

FIGURE 1
Comparison of the changes in the Epidermal Mitotic Index (E.M.I.) between control and cortisone treated animals.

The connective tissue contains many fibroblasts and fibrocytes as well as other connective tissue elements. At the cut surface, due to the severing of the blood vessels, many erythrocytes and scattered leucocytes are found.

Four days after amputation

During the first four postoperative days, the epidermis plays an important role in the closure of the amputation wound. The epidermal cells from the stump close the wound by the combined processes of cell stretching and cellular proliferation. This wound closure by "secondary intention" (Needham, '52) is characteristic of aquatic vertebrates. The migrating cells are replaced by an increased epidermal mi-

totic activity. In the normal animal, this epidermal mitotic index has increased from 0.23 to 0.41%. The resulting epidermal closure is approximately 4 to 5 cells thick. Due to the replacement by epidermal mitotic activity, the epidermis has maintained its normal thickness of 3 to 4 cells.

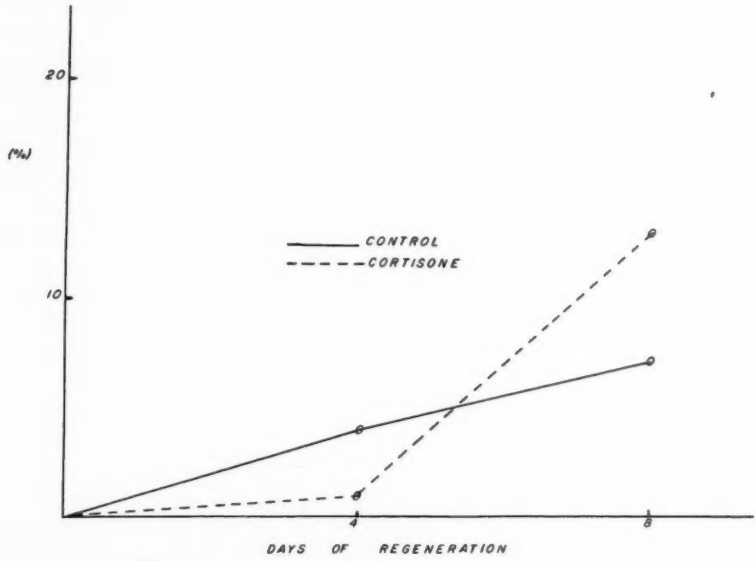

FIGURE 2

Comparison of the changes in the Mesenchymal Epidermal Ratio (M.E.R.) between control and cortisone treated animals.

In those animals which received daily intramuscular injections of cortisone acetate, the mitotic activity of the epidermis was inhibited. The mitotic index has dropped to 0.17% which is below the replacement mitotic index of the normal limb. Consequently, any wound closure due to epidermal migration will result in a thinning of the stump epidermis. The wound closing epidermis of the cortisone treated animal at 4 days is an average 2 to 3 cells thick. In addition, there is an abundance of intercellular matrix. The stump epidermis has thinned. In most regions of the stump it is only 1 to 2 cells thick.

The mesenchymal mitotic activity has increased slightly, from 0.00% to 0.04%. These cells will eventually migrate between the cut end of the bone and the overlying epidermis (Manner, '53). This increase in mesenchymal mitotic activity is also evidenced in the cortisone treated animals but to a lesser extent, from 0.00 to 0.01%.

Eight days after amputation

The migration of new cells over the wound surface has continued, although the mitotic activity of the epidermis has begun to return to normal. The epidermis in the wound area is now approximately 18 cells thick. The stump epidermis continues to remain at a thickness of 3 to 4 cells.

Since the last stage, the blastema has begun to form in the normal limb. In its earliest stages, this is identified as a migration of connective tissue cells between the cut end of the bone and the overlying epidermis. This increase in volume of connective tissue cells which allows for this blastema formation is due to the increased mesenchymal mitotic activity. From an initial 0.00% it has now become 0.07%.

The cortisone treated animals now present a more normal picture, although they are approximately four days retarded in their development. The epidermal wound covering is much thicker now and the epidermis in the rest of the limb has been restored to normal. The epidermal mitotic index is now 0.62%, almost twice that of the control animals.

The connective tissue is also in a stage similiar to the four day control animal. The mesenchymal mitotic activity has increased from 0.00 to 0.13%. However, not enough new connective tissue has formed to permit blastema formation.

Discussion

A—Mechanism of action

The closing of the amputation wound is dependent upon two epidermal activities, i.e., mitosis and cell migration. From the data presented, it appears that cellular migration is not inhibited. The thinness of the epidermal wound covering is due to a lack of new cells, not to the inability of the epidermal cells to migrate. A sharp drop in epidermal mitotic activity is noted in those animals which were treated with cortisone acetate.

There does not appear to be any specificity of the cortisone acetate for a particular tissue, as the mitotic activity of both the epidermis and mesenchyme are inhibited. There does, however, appear to be a direct correlation between the initial rate of mitotic activity and the degree of inhibition. The epidermis, having a higher normal mitotic index, is more inhibited than the mesenchyme.

Cortisone is known to exert an inhibitory action on carbohydrate utilization. Perhaps the inhibitory effect on mitotic activity can be ascribed to the smaller quantity of energy available to the cell due to this inhibition of carbohydrate utilization brought on by cortisone acetate.

B—Homeostatic adjustment

A striking phenomenon presents itself eight days after amputation. The mitotic activity of the epidermis and mesenchyme of the cortisone treated animal is higher than that in the control animal. It appears that the initial inhibitory effect of the cortisone acetate has been neutralized and that now, freed from inhibition, the retarded tissues are attempting to "catch up" with the controls and consequently, have a higher rate of mitotic activity. This phenomenon of accelerated growth after the removal of an inhibitory agent is well known. The factors responsible for this neutralization are not known at present.

In vertebrate organisms, there is a tendency to maintain a constant titer of materials circulating in the blood stream. This maintenance of a constant level, referred to as homeostasis, is exhibited in the changes in hormone concentrations in the blood stream.

In the present case, 0.0005 mg. of cortisone acetate was injected daily. If this hypothesis is correct, this amount may be assumed to be less than the total daily output of the adrenal cortex. In compensating for this daily injection, the gland would decrease its daily output by approximately .0005 mg. In this way, the hormone concentration in the blood stream would remain normal. It evidently requires between four and eight days to accommodate in this manner.

Summary

1. The right forelimb of the newt, *Triturus viridescens viridescens* (Rafinesque) was amputated. The experimental animals received injections of .0005 mg. of cortisone acetate daily.

2. An inhibitory effect of cortisone acetate on wound healing was noted. As a result of mitotic counts, it is postulated that the action of the hormone is not on the cells' migratory activity but on the mitotic activity of both epidermal and mesenchymal cells.

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THE RELATION OF BLOOD GLUTATHIONE LEVEL TO GROWTH AND BODY SIZE IN BEEF CATTLE[1]

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Rate and economy of gain and body size are important selection criteria in the breeding and improvement of beef cattle. The efficiency of a selection program could be greatly increased if an animal's potential performance for these traits might be evaluated early in life. It appears reasonable to expect that variation among animals within a species with respect to these traits is the result of variation in fundamental metabolic processes. It is possible that such variation might be reflected, at least in part, by differential amounts of one or more metabolites measurable at an early age. Evidence is available indicating that glutathione might be such a metabolite. The purpose of this investigation was to determine whether there existed a relation, amenable to available measurement techniques, between the blood level of glutathione and body size and subsequent rate and economy of gain of long yearling steers.

LITERATURE

A comprehensive review of the literature dealing with glutathione is beyond the scope of this paper. Rather, attention is called to the Proceedings of a recent Symposium on Glutathione (Colowick, 1954) and some papers pertinent to this investigation.

Painter (1928) found that at 9 and 12 days embryos of large race rabbits and of hybrids of large and small races had a higher rate of cell proliferation than embryos of small races, but there was no difference in cell size.

Castle and Gregory (1929) and Gregory and Castle (1931) found

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that during embryonic growth eggs of large race rabbits segment at a significantly greater rate than those of small races. Castle (1929, 1931, 1932) showed that rate and duration of growth during postnatal development were greater in large race rabbits than in small races. Blunn and Gregory (1935) found that embryos of a heavy poultry breed proliferated cells at a greater rate than embryos of a lighter breed. These studies indicate that hereditary growth rate and size are related to rate and duration of cell proliferation.

Hammett (1929a, 1929b, 1930a, 1930b, 1932, 1934) demonstrated a relation between sulfhydryl—SH—and growth, particularly between SH and rate of cell proliferation, in a variety of biological test groups. Hammett and Hammett (1932a, 1932b, 1932c, 1933); Hammett, Green and Davenport (1932); Voegtlin and Chalkley (1930); Chalkley and Voegtlin (1932); White (1933); Voegtlin (1934), and Coldwater (1933) have confirmed Hammett's original work. Rapkine 1931) was able to determine free SH at various stages in the first division cycle of the sea urchin, *Paracentrotus lividus*. The free SH decreased during the first stages then increased to a level above the initial value. The peak value coincided with cleavage of the egg.

Gregory and Castle (1931), on the basis of Hammett's early work, suggested the possible role of SH in controlling rate and duration of cell proliferation, and hence adult size.

Voegtlin, Maver and Johnson (1933a, b) reported that the SH group attached to protein or in the form of glutathione—GSH—which can give rise to a relatively high concentration of disulfides, is one of the factors which favor the synthesis of proteins.

Wilkerson and Gortner (1932) concluded that GSH is of vital importance in tissues of the embryonic pig and is in some way associated with protein metabolism and synthesis.

Gregory and Goss (1933a, b, c) presented evidence that the GSH concentration of body tissues of non-nursed rabbits 48 hours of age is associated with the genetic constitution for adult size and rate of segmentation of eggs. Gregory, Goss and Asmundson (1935, 1936, 1937); and Gregory, Asmundson, Goss and Landauer (1939) have shown a similar relation in chicken embryos and early hatched chickens.

These studies indicate that GSH is associated with rate of cell proliferation, protein synthesis and adult size. It does not necessarily follow that there is a cause and effect relationship. However, the

evidence is conclusive that there is an association between GSH concentration and rate of cell proliferation, protein synthesis and adult size.

The effect of certain environmental factors has been demonstrated. Goss and Gregory (1932, 1935a) showed that nursing approximately doubled the concentration of GSH in the entire body of the young rabbit. Gregory and Goss (1934, 1939) and Goss and Gregory (1935b) found that in rats and rabbits changes in GSH concentration were associated with response to injection of hypophyseal growth hormone. Chanton (1932) reported that in the domestic fowl, the blood of males has a significantly greater GSH concentration than the blood of females. Gregory and Goss (1933b) reported no effect of sex on GSH concentration in the total carcass of non-nursed rabbits at 48 hours.

Kunkel, Stutts and Shrode (1954) reported a significant lower level of blood GSH in the Hereford breed of cattle than in the Angus or Brahman breeds. Their data indicated possible sex differences, but the authors did not elaborate. Reid, Ward and Salsbury (1948) found a gradual increase in bovine blood GSH with increasing age. However, Kunkel, et al. (1954) concluded that differences in GSH levels in their data were not the result of age. Kunkel, et al., obtained a heritability estimate of 0.86. This would indicate that mass selection would be quite effective in increasing the blood level of GSH.

Briefly, the rationale leading to this study includes the notion that growth rate and adult size are determined by rate and duration of cell proliferation, which involves protein synthesis. There is an association, the exact nature of which is unknown, between GSH concentration and rate and duration of cell proliferation and protein synthesis. This relation may be modified by certain environmental factors, but heritability appears quite high. It seems entirely possible, therefore, that the level of blood GSH in beef cattle might be associated with subsequent rate and economy of gain and with body size, and that early selection for increased blood GSH would be effective in improving these traits.

Materials and Methods

The animals used in this study consisted of 47 range steers 15 to 21 months of age. An attempt was made to obtain equal numbers of 5 grades of feeder steers, but within each grade there was no selection

of animals. The steers were assembled at the experimental farm and allowed several weeks to become acclimated to the ration, pens and feeder. During the experiment the steers were individually fed in small pens twice a day, but were allowed to run together in groups of 10 to 12 at other times. The ration consisted of a grain concentrate and grass and clover hay. Each animal was allowed all it would eat, but an attempt was made to maintain approximately a 1:1 concentrate to roughage ratio.

At the start of the trial the steers were weighed, measured and blood samples obtained following a 12-hour fast. The blood samples were drawn from the right jugular into tubes containing sodium citrate as the anticoagulant. Except for one group, all blood analyses were completed within ten hours. The exceptional group was analyzed within 18 hours. GSH was determined in whole blood by the method of Grunert and Phillips (1951). Hemoglobin was determined colorimetrically as oxyhemoglobin. Total cell count was determined indirectly by turbidity at a dilution of 1:333 in normal saline, and read at a wave length of 700 mu.

At the completion of an 84 day feeding period blood samples were again taken and analyzed as described. A longer feeding period may have been desirable, but was precluded by circumstance. The data were amenable to analysis of variance and correlation techniques as described by Snedecor (1946).

Results and Discussion

Glutathione was determined in duplicate from a single blood sample, and an average of the two values was used in all calculations. Preliminary analysis indicated small error. There was no significant difference between duplicates, and the correlation between them was 0.99. It seems reasonable to conclude that the chemical method is quite accurate.

Relation of GSH to size, rate and economy of gain

The essential correlations between the level of blood GSH and body size, and rate and economy of gain are presented in table 1. The correlation between GSH and body weight at the start of the trial was not significant, nor was the correlation between GSH level and weight at the conclusion of the trial. There was a highly significant correlation between GSH level and heart girth at the start of the trial. Heart

girth was not determined on all animals at the conclusion. The significant correlation with heart girth but not with weight is in agreement with Kidwell's (1954) suggestion that heart girth is a more suitable measure of total size than weight for studies of growth and form. Gregory and Goss (1933b) found no relation between the weight of the newborn rabbit and the GSH concentration of the entire carcass. However, Gregory and Goss did not compute a correlation coefficient, and their data were from a small, rather uniform population. It is also possible that carcass weight is not the most suitable measure of size in the newborn rabbit.

TABLE 1
Correlations between blood level of GSH and size, rate and economy of gain.

	Initial weight	Initial heart girth	84-day gain	#grain/ #gain	#hay/ #gain	Total grain consumed	Hb final	Total cell final
Initial GSH	0.21	0.42**	—0.28	0.35*	0.33*	0.19	—	—
Final GSH	—	—	0.16	—0.20	—0.19	—	0.58**	0.48**

* 5% level of significance.
** 1% level of significance.

These data indicate no relation between GSH level and subsequent or previous rate of gain. Further, there appears to be no relation between GSH level and previous economy of gain. This is in agreement with the conclusion of Kunkel, et al. (1954). There is, however, a small but significant relation between GSH level and subsequent economy of gain. Although this correlation is probably too low to be of value in a selection program, it indicates the possible existence of measurable metabolites that might bear a higher relation to this trait. Kunkel, et al. (1954) reported no evidence of such a relation. However, they did not elaborate on their data.

Glutathione is known to be associated with cell proliferation, and possibly enlargement (Mazia 1954). It is quite likely that animals gaining most economically were for the most part increasing muscle tissue and those gaining less economically were largely depositing fat. This could provide a physiological basis for the observed relation.

The correlation between initial GSH level and total grain consumed was calculated as an indication of a possible relation between GSH level and appetite. No such relation was observed.

Relation of GSH to Hemoglobin and Total Cell Count

A highly significant correlation of 0.58 was observed between GSH

and hemoglobin. This is similar to the correlation of 0.50 observed in Hereford cattle by Kunkel, et al. (1954). The observed correlation between GSH and total cell count was 0.67. This is in agreement with reports in the literature, and indicates these samples were not unusual. These data indicate that variation in total cell count or hemoglobin may account for 23 to 30 per cent of the variation in GSH. This is in agreement with the findings of Kunkel, et al. (1954).

The correlation between blood GSH and hemoglobin (0.58) and between blood GSH and total cell count (0.48) indicates that a large fraction of the blood GSH is found in the red blood cells, and the remainder in the plasma. It is possible that the GSH in the red blood cells is in depot form and that in the plasma in a dynamic state. If this were true, the correlation between plasma GSH and rate and economy of gain would likely be higher than the correlation between whole blood GSH and these traits. An experiment has been designed to test this suggestion.

Repeatability

The mean value of GSH at the start of the trial was 24.18 ± 0.79 mg. per cent and the coefficient of variation 0.22. The mean value at the conclusion of the trial was 31.54 ± 0.72 mg. per cent and the coefficient of variation 0.15. The difference between the means is highly significant. The correlation between the two determinations on the same animal taken 84 days apart is 0.36, a significant value. The means, standard errors and coefficients of variation are in general agreement with those reported by Kunkel, et al. (1954). The correlation between determinations, taken 84 days apart, however, is much lower than the values of 0.74 to 0.97 reported by these authors. The animals studied by Kunkel, et al., may have been on a fairly uniform high plane of nutrition prior to both determinations, while those cited here were on a low plane of nutrition prior to the first determination, but on a high plane prior to the second. These differences may account for the discrepancy. It is also possible that additional undetected environmental factors effected these results.

Summary

Blood level of glutathione, weight, heart girth, hemoglobin, total cell count, and rate and economy of gain during an 84-day feeding trial was determined for 47 Hereford steers. The analyses indicate:

1. There is no relation between blood GSH and weight, or rate of gain.
2. There is a small correlation between blood GSH and subsequent economy of gain and between blood GSH and heart girth.
3. The blood level of GSH is correlated with hemoglobin and total cell count.
4. There was a significant difference between the blood level of GSH at the beginning and end of the 84-day period. There was a small correlation between blood GSH determined at the beginning of the trial and at the conclusion. It is suggested that differences in the plane of nutrition may be important.
5. It is concluded that the level of whole blood glutathione is not useful in a selection program for rate and economy of gain and body size. However, the small correlation between GSH and economy of gain indicates the possible existence of suitable metabolites.

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STUDIES ON THE PROTEIN COMPOSITION OF ALCOHOLIC EXTRACTS OF RAT SARCOMA AND SIMILAR EXTRACTS OF NORMAL MUSCLE TISSUE*

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Previous studies (28) on the amino acid composition of alcoholic extracts of rat sarcoma and similar extracts of normal muscle tissue revealed both quantitative and qualitative differences in the two types of extracts. For example, a large part of the nitrogen in both extracts was found to exist in the form of free amino nitrogen, which was twice as great in the tumor as in the normal tissue extracts. An increase in the free amino nitrogen of both extracts after hydrolysis with hydrochloric acid indicated that some protein or peptide was present. Also, the appearance of the patterns obtained by starch column chromatographic analysis showed that the extracts contained some protein or peptide. By this technique the kinds of amino acids which increased after hydrolysis of the tumor extract were found to be different from those in the normal tissue extract, indicating that the proteins or peptides must be different in chemical composition. Such differences in protein composition might prove to be of extreme importance in view of the reported oncolytic and immunizing properties of the extracts (2, 3, 4).

Differences and similarities in the proteins of tumor and normal tissues have been found by other workers (23, 26, 30, 31, 32), and the protein aspects of cancer have been reviewed and discussed by Toennies (34, 35, 36). The earlier chemical studies, however, were carried out on tissue extracts prepared with various types of salt solutions. The extractions yielded numbers of proteins of large molecular weight, which would not necessarily be extracted in alcohol. Al-

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coholic extracts of tumor tissue have been found to produce antibodies (7, 37) but no attempt was made to associate the antigenic properties with any particular substance in the extracts.

These studies, therefore, were designed to determine the nature and amount of the protein present in the extracts, and whether they are capable of exhibiting antigenicity. For this purpose, the extracts were dialyzed, determination of the amount of dialyzable and non-dialyzable nitrogen was made, and the fractions obtained were examined by the electrophoretic technique. The whole extracts were injected into rabbits to test their ability to produce antibodies, and the presence of antibodies was detected by qualitative precipitin tests using the extracts as antigens. In order to determine whether the proteins present were responsible for antigenicity, the dialyzable and non-dialyzable fractions of the extracts were also used as antigens in the precipitin test. For comparative purposes an extract of a lymphoma which arose spontaneously in the same inbred strain of rats was also studied.

Materials

Source and preparation of the extracts. All of the rats used in this study were from a strictly inbred Wistar strain.[1] Two types of 100 per cent transplantable neoplasms native in this strain, the Lewis sarcoma No. 10 and the Lewis lymphoma No. 8, were used. Descriptions of these tumors are found elsewhere (3, 13, 29). Normal muscle tissue was obtained from rats of the same strain.

The method of extraction of the tissues was modified slightly from the original procedure (2, 3) in order to obtain preparations suitable for chemical analysis. The details of this modified procedure are described in a previous publication concerning the gross chemical composition of the extracts (29).

Chemical Methods

Fractionation of the extracts by dialysis. The extracts were separated into protein and non-protein fractions by dialysis. These experiments were carried out on aliquots of the extracts which were dried *in vacuo* to remove the alcohol. The dried material was put back into solution in the least amount of distilled water necessary to dissolve it and dialyzed in cellophane tubing against distilled water

[1] Tumor bearing and normal rats were kindly given to us by Dr. Margaret Reed Lewis.

at 3 to 4° C. The dialysis was first carried out in 5-6 portions of distilled water only two to three ml. greater than the volume of the sample being dialyzed. The dialysates were combined, dried *in vacuo*, and redissolved in the smallest volume of distilled water possible. After aliquots were removed for nitrogen determinations, the dialyzed material was redialyzed against approximately 200 times its volume of distilled water in order to remove all traces of dialyzable material. The amount of nitrogen lost in dialyzing against the larger volume was calculated by finding the difference in the non-dialyzable nitrogen after the first and second dialyses.

Nitrogen determinations. The dialyzable and non-dialyzable nitrogen, as well as the total nitrogen, in the extracts was determined by a micro-Kjeldahl nesslerization procedure (22), modified to be used at a level of 10 gamma of nitrogen (10). The protein concentration of the non-dialyzable fraction was calculated by multiplying the nitrogen values by the factor 6.25.

Ninhydrin determinations. Quantitative colorimetric ninhydrin analyses were made by the revised method of Moore and Stein (25).

Electrophoresis. Electrophoresis experiments were carried out in a paper strip model electrophoresis apparatus obtained from Arthur H. Thomas Company. All experiments were run in pH 7.38 phosphate buffer of 0.1μ.

For paper electrophoresis the samples were applied on a vertical line to 1¼ or 2½ inch strips of Whatman No. 1 filter paper. At the end of the experiment the strips were dried in an oven at 90°C. for 15 minutes. The color was developed either by spraying with 0.2 per cent ninhydrin in normal butanol saturated with water and drying at 90°C. for 5 minutes or by staining with Bio-blue and washing with 1:1 methanol-glacial acetic acid.

The paper electrophoresis apparatus was also adapted for starch block electrophoresis described by Kunkel (11) by substituting a power pack of 300 V capacity for the one supplied with the apparatus.[2] Eimand starch was washed free from ninhydrin reacting materials, moistened with buffer and made into a block 9¼ by 1¼ inches held together between two glass plates. At the end of the experi-

[2] This modification in the apparatus was kindly made for us by the Arthur H. Thomas Co.

ment the block was cut into centimeter portions, which were eluted with 0.05μ phosphate buffer. The buffer was diluted to this ionic strength to make possible nitrogen determinations by nesslerization, which was the method used for obtaining the concentration of material in the eluted fractions.

Immunological Procedures

Production of antibodies. The extracts were tested for their ability to produce antibodies by injection into rabbits. Commercially available male rabbits weighing from 2100 to 2600 grams were used. For these injections the extracts were dried *in vacuo* to remove the alcohol and rediluted with buffered saline on the basis of the protein concentartion estimated from the amino nitrogen values (28). This dosage contained the equivalent of 0.81 mg. of protein. In the earlier experiments smaller amounts of protein were given because the actual protein concentrations were not yet known. In most instances the first injection was given intravenously, followed by subsequent intracutaneous injections at weekly intervals.

An adjuvant was also employed as used by Suter (33). A mixture in the proportion of 0.1 ml. of antigen in buffered saline, 0.1 ml. Arlacel and 0.3 ml. Bayol F containing 100 γ protein in 0.1 ml. was injected intracutaneously in 0.2 or 0.4 ml. amounts. When one injection did not produce antibodies, another injection was given two to three weeks later.

Blood was obtained at intervals during the experiments from a slit in the marginal ear vein, and the serum was separated and stored at —6°C.

Detection of antibodies. Qualitative precipitin tests were used for detecting the presence of antibodies in sera of the rabbits which had been injected with the extracts. These tests were made in 6 x 50 mm. tubes using 0.1 ml. of the extracts or fractions of extracts diluted serially as antigens. Phosphate buffer or 0.85 per cent saline to which merthiolate was added were used as diluents. Mixtures of the serum and antigen were incubated for 2 hours at 37°C. and allowed to stand overnight in the cold at 3 to 4°C. before readings were made. A second reading was made after four to five days.

Chemical Results

Analyses for dialyzable and non-dialyzable nitrogen. The values obtained for the dialyzable and non-dialyzable nitrogen and the protein concentrations, calculated by the use of the factor 6.25, are given in Table I. It may be seen from this table that the protein concentration was six times as great in the sarcoma extract, 0.325 mg./ml. and four times as great in the lymphoma, 0.209 mg./ml., as in the normal tissue extract, 0.052 mg./ml. The non-dialyzable nitrogen, on which the protein concentrations were based, represented only a small fraction of the total nitrogen of the extracts; 2.7-2.9 per cent in the sarcoma, 1.7-1.8 per cent in the lymphoma and 0.24-0.29 per cent in the normal tissue extract. The values for the dialyzable nitrogen and the total amount of nitrogen recovered are also given in this table.

Electrophoretic analyses. Since the paper electrophoresis technique is adaptable to the separation of small molecules, such as amino acids and peptides, as well as proteins, both the dialyzable and non-dialyzable fractions of the extracts were studied by this method. The paper strips were stained with two different reagents, namely, Bio-blue, which gives a deep blue color with proteins but no color with amino acids, and ninhydrin, which gives a strong violet color with amino acids and stains proteins faintly.

Paper strips on which the dialyzed fractions were run, when sprayed with ninhydrin, gave patterns identical with those found previously for the original extracts (28). The non-dialyzable fractions were concentrated by evaporation to dryness in a vacuum dessicator, and then they were resuspended in buffer in order to obtain a sufficiently high protein concentration for paper electrophoresis experiments. Duplicate strips of each preparation were run simultaneously, after which one was stained with Bio-blue and one with ninhydrin. Diagrammatic drawings of these strips showing the position of the components after 2½ hours electrophoresis in 0.1μ phosphate buffer at pH 7.38 are shown in Figure 1, and an attempt was made to demonstrate differences in their relative densities by variations in stippling.

Paper strips on which the protein fraction from the normal extracts was run from 1-5 hours showed no components when stained with Bio-blue, but when sprayed with ninhydrin one heavy and three very faint components were visible. The dialyzed sarcoma extract,

TABLE I
Comparison of the Dialyzable and Non-Dialyzable Nitrogen and Protein Concentration of Extracts of Sarcoma, Lymphoma and Normal Tissue

Extract	Total Nitrogen	Dialyzable Nitrogen		Non-Dialyzable Nitrogen		Per Cent Nitrogen Recovered	Protein (Based on Non-Dialyzable Nitrogen)	
	mg/ml	mg/ml	% of Total N	mg/ml	% of Total N		mg/ml	Ave.
Sarcoma No. 10	1.81	1.60	88	0.048	2.7	91	0.304	
"	1.81	1.60	88	0.055	2.9	91	0.344	
"	1.81	2.04	112	0.053	2.9	115	0.331	
								0.325
Lymphoma No. 8	1.90	1.85	97	0.032	1.7	99	0.199	
"	1.90	1.83	96	0.035	1.8	98	0.219	
								0.209
Normal Tissue	3.33	3.17	95	0.0081	0.24	95	0.0506	
"	2.98	3.32	112	0.0084	0.29	112	0.0525	
"	2.98	3.24	109	0.0085	0.29	109	0.0533	
								0.0521

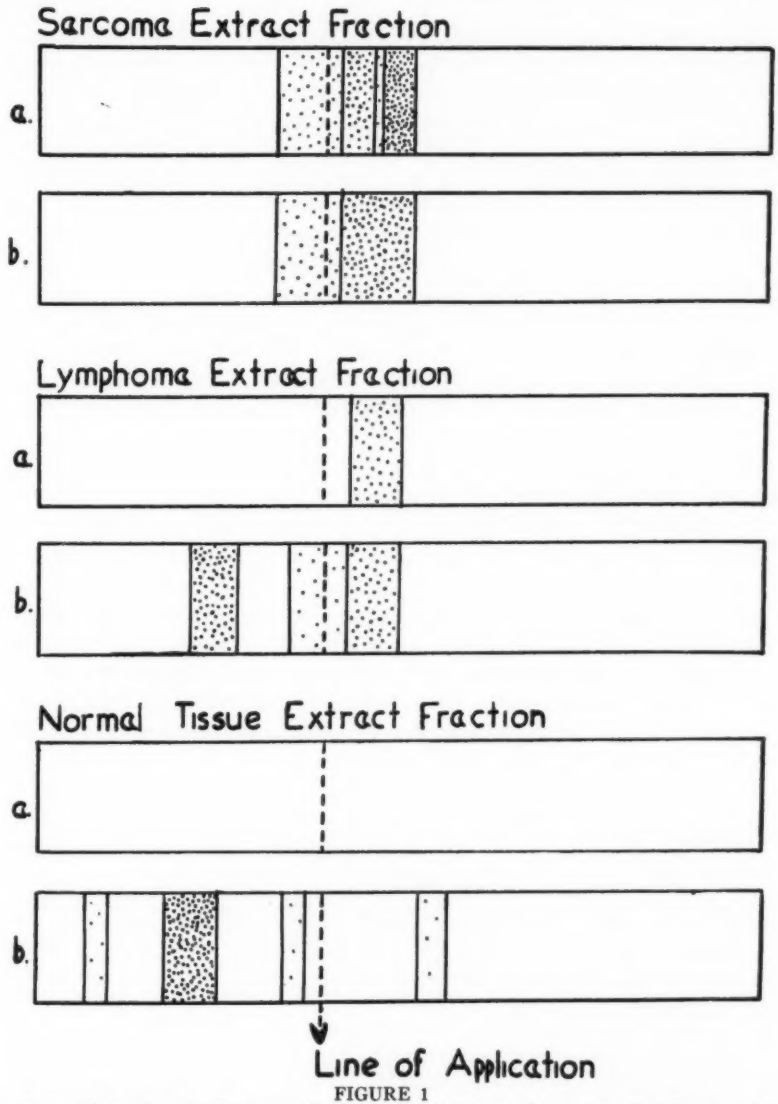

FIGURE 1

Paper electrophoretic diagrams of the protein fractions of sarcoma, lymphoma and normal tissue extracts stained with Bio-blue (a) and ninhydrin (b). Phosphate buffer, pH 7.38, 0.1μ, for 2½ hours at 25 milliamperes.

when stained with Bio-blue showed the presence of three slow moving, electrophoretically distinct components, which were not well separated from one another. These components also stained with ninhydrin. The electrophoretic pattern of the protein fraction of the lymphoma extract, when stained with Bio-blue, revealed the presence of only one component. However, when stained with ninhydrin, two additional components were visible, one of which appeared to have a positive electrophoretic mobility. This component had a similar position to the major ninhydrin staining component of the normal tissue fraction.

The small amount of protein present in these extracts prevented using the Tiselius electrophoresis technique for analysis. However, it was possible to prepare enough of the dialyzed material from the sarcoma extract to attempt fractionation by starch block electrophoresis. By this method, as was found by paper electrophoresis, the sarcoma extract was shown to contain three distinct proteins. The separation was not completely clean, however, even though the experiment was carried out for a much longer time than by paper electrophoresis.

Quantitative analyses for ninhydrin color values. The differences found in the ninhydrin staining properties of the components separated by paper electrophoresis were of great interest, since not only do different amino acids exhibit slightly different color values, but also considerably lower values are given by progressively larger molecules, such as the peptides and proteins. Thus the degree of breakdown of proteins can be roughly estimated in this manner. Therefore, the quantitative colorimetric ninhydrin method was used to study the nondialyzable fractions of these three extracts, and the amount of color produced was converted into the milligram equivalents of leucine and related to the protein nitrogen of the extract. Determinations were

TABLE II

Comparison of the Ninhydrin Color Producing Capacities of the Protein Fractions from the Sarcoma, Lymphoma and Normal Tissue Extracts with That of Crystalline Bovine Serum Albumin.

Materials Analyzed by Ninhydrin Method	Color Yield*
Crystalline Bovine Serum Albumin	0.047
Protein Fraction from Sarcoma No. 10 Extract	0.059
Protein Fraction from Lymphoma No. 8 Extract	0.066
Protein Fraction from Normal Tissue Extract	0.115
Leucine	1.00

* The color yield was related to the nitrogen concentration of the preparations.

also made on crystalline bovine serum albumin for comparison. The color yields are shown in Table II, where it may be seen that the sarcoma and lymphoma fractions have color yields of 0.059 and 0.066, respectively, which approach that of crystalline bovine serum albumin, 0.047. The color yield for the normal tissue fraction was 0.115 indicating that it is a smaller molecule.

Immunological Data

Reaction of rabbits to injection of extracts. The extracts of sarcoma, lymphoma and normal tissue were injected into rabbits in order to test their ability to produce antibodies. Antibodies to both tumor extracts were obtained, but none to the normal tissue extract. Before injection into the rabbits, the alcohol was removed by evaporation to dryness *in vacuo,* and the extracts were then resuspended in buffered saline. However, when enough of the normal tissue extract was used to make the protein concentration equal to that of the tumor extracts, the first injection, which was given intravenously, killed the rabbit. Therefore, this extract was dialyzed to remove the salts, and only the non-dialyzable or protein fraction was used.

Striking differences were observed in the skin reactions of the rabbits to the injections of the tumor and normal tissue extracts. Both the sarcoma and lymphoma extracts gave an Arthus type reaction with swelling, induration and central necrosis, which increased in severity with each injection. The skin reaction to the normal extract was slight, with little swelling or induration, and, even when the protein fraction was given in an adjuvant, no necrosis was evident.

Precipitin tests with extract antigens. Precipitin tests were carried out on sera obtained from these rabbits, using as antigens the extracts which had been evaporated to dryness *in vacuo* to remove the alcohol and rediluted with 0.85 per cent NaCl on the basis of the protein concentration of the extract. The relative amounts of original solution contained in the antigen are indicated in Table III, which shows the precipitin reactions of these antisera when the titre was highest. It is evident that antibodies were obtained to both the sarcoma and lymphoma extracts, but none to the normal extracts.

The data shown here are taken from readings which were made after the precipitin tubes had remained for 4 to 5 days at 4°C. The precipitates were appreciably heavier at this reading than at the one

TABLE III

Precipitin Reactions of Sera of Rabbits Injected with Sarcoma, Lymphoma and Normal Tissue Extracts Using the Whole Extracts as Antigens.

Rabbit Number	Extract used for Injection into Rabbits	Extract used for Antigen in the Precipitin Test	Ml. of Original Extract per 50 γ of Protein	Antigen Protein in Precipitin Test Gamma of Protein Used in Test							
				50 γ	20 γ	10 γ	5 γ	2 γ	1 γ	0.5 γ	0.2 γ
9	Sarcoma No. 10	Sarcoma No. 10	0.16	+++++	++	+++G	++++G	+++++G	+++G	+	0
22	"	"	"	++++	++	+	+	++G	+	0	0
37	"	"	"		+	0	+++G	++++G	++G	+G	0
39	"	"	"	++++	++	+	0	±	±	0	0
24	Lymphoma No. 8	Lymphoma No. 8	0.26	++++	+++G	+++++G	++G	+	+	0	0
34	"	"	"	+++	++	+	0	0	0	0	0
36	"	"	"	++++	++G	+++G	++	±			
13	Normal Tissue	Normal Tissue	0.69	±	0	0	0	0	0	0	0
30	"	"	"	0	0	0	0	0	0	0	0
38*	"	"	"	+	0	0	0	0	0	0	0
9	Sarcoma No. 10	Lymphoma No. 8	0.16	+++++	++G	+++G	++++G	±	±	0	0
9	"	Normal Tissue	"	+	±	0	0	0	0	0	0
24	Lymphoma No. 8	Sarcoma No. 10	0.26	++++++	+++	±	++++G	+++G	++	±	±
24	"	Normal Tissue	"	0	0	0	0	0	0	0	0
38*	Normal Tissue	Sarcoma No. 10	0.69	++++	++	0	0	0	0	0	0
38*	"	Lymphoma No. 8	"	+++	0	0	0	0	0	0	0

* Rabbit Number 38 was injected with dialyzed Extract.

made at 24 hours. The sera of rabbits No. 22, 39 and 36 showed definite precipitates at the second reading, whereas, on the first reading weak precipitates or none at all were found. The sera of rabbits No. 9, 37 and 24 reacted strongly on the first as well as the second reading. The heavy precipitates showed a strikingly granular quality and did not disperse readily on shaking. This characteristic is designated in all tables as "G".

The precipitates described above appeared in the sera only after eight or more injections of the rabbits with the tumor extracts. However, there was still another substance in these antisera, as well as in normal rabbit sera, which gave a precipitate with the higher concentrations of the tumor antigens, and this latter precipitate dispersed readily on shaking and did not have the granular quality of the precipitate mentioned above. This reaction was strong with the antigen dilution containing 50 γ protein, and it decreased sharply in the next dilution, even to negative in many cases. The normal tissue antigen gave only a trace of precipitate in these higher antigen concentrations. The distinction between the two precipitates was more striking at the time of the first readings of the precipitin reactions than is shown in the tables, in which the second readings are recorded. At the second reading some overlapping occurred, especially with the lymphoma extract antigen.

In order to determine whether the antibodies formed to the sarcoma and lymphoma extracts would show cross reactions, precipitin tests were carried out using all possible combinations of antisera and antigens. The reactions obtained (also given in Table III) showed that the antibodies to the sarcoma crossed with the lymphoma antigen, as did those of the lymphoma with the sarcoma antigen, while neither showed any cross reaction with the normal tissue antigen.

The course of the antibody production was studied in greatest detail in the first rabbit, No. 9, which was injected with the sarcoma extract. Table IV shows the precipitin reactions of the sarcoma extract with the serum of this rabbit taken 3-26 days after injection. Antibodies appeared in the serum after 8 injections and reached a five plus reaction after 13 injections. The table shows that when injections were discontinued the antibody response fell off rapidly to a weak or negative reaction, and usually more than one injection was necessary to produce a strong reaction again. The later injections were given in an adjuvant with the hope of maintaining a high antibody

TABLE IV

Precipitin Reactions of the Sera from Rabbit No. 9 and the Sarcoma Extract Showing the Rise and Fall of the Antibody Titre During Various Intervals of Injections.

Number of Injections	Days after Injection of Bleeding	Gamma of Antigen Protein in the Precipitin Test							
		50 γ	20 γ	10 γ	5 γ	2 γ	1 γ	0.5 γ	0.2 γ
0	0	++++	0	0	0	0	0	0	0
5	2			±	0	0	0	0	0
8	5			0	+++G	++G	±	+	±
11	6			++	+++G	+++G	++G	++	+
13	3		±	++	+++G	++++G	+++G	±	0
13	14		±	0	++	+++G	++	++	+
13	35		±	0	0	0	0	0	0
14	6	++++	±	0	±	++G	±	±	0
14	12		0	0	0	0	+G	0	0
15	5	+++	0	0	0	±	+	±	0
16*	6		±	++G	+++G	++++G	+	±	0
16	12		+	±	++++G	++++G	++G	±	±
16	18		±	±	±	±	0	0	0
16	26		0	0	0	0	0	0	0
17	4		++	0	0	0	0	0	0
17	11		0	0	0	0	0	0	0
18	10		++	0	++++G	+++	0	0	0
18	13		++	0	++	+++	+	0	0
19	6		0	±	+++G	+	+	±	0
19	13	++++	0	±	++G	+++G	+	0	0

* All injections after the 16th were given with an adjuvant.

titre for a longer period of time, but even in this case antibodies disappeared from the serum soon after injections were discontinued.

Precipitin tests with fractions. Precipitin tests were also carried out with dialyzable and non-dialyzable fractions obtained from the sarcoma and normal tissue extracts and the antisera to these extracts. The dialyzed fraction was diluted on the basis of protein concentration. The dialyzable material contained no protein, and, therefore, it was diluted to a concentration equivalent to the nitrogen content of the original extracts used in the test, in order that the salt concentration would be similar to that of the whole extract which had been used as an antigen. The data obtained from these tests are shown in Table V, and it is apparent the material which reacted with the antibody to the sarcoma extract was found in the non-dialyzable fraction, indicating that it was a protein. Although not shown in the table, the proteins separated by starch block electrophoresis of the sarcoma extract were also used as antigens in the precipitin test with the serum containing antibodies to the whole sarcoma extract. All three proteins reacted with this antiserum, showing that antibodies had been produced to each of them.

Table V also shows that the substance responsible for the precipitin reaction, which was obtained with normal as well as immune rabbit serum, is located in the dialyzable fraction. This substance, therefore, is non-protein in nature and in no way related to the material which precipitates the antibodies arising after immunization with the extracts, which is found in the non-dialyzable fraction.

Discussion

Striking differences, both quantitative and qualitative, have been found in the protein composition of alcoholic extracts of tumor tissue and extracts of normal tissue similarly prepared. Such differences are not surprising, since protein synthesis and breakdown are under the control of enzyme systems, which have been found to be consistently different in normal and neoplastic tissues. For example, Maver (18, 19, 20, 21) isolated the cathepsins, which are endocellular proteinases, from transplanted rat tumors and normal rat tissue and demonstrated by serological techniques that they are different proteins.

In the present work it was found that there was six times as much non-dialyzable nitrogen in the sarcoma extracts as in the normal

TABLE V

Precipitin Reactions of Sera of Rabbits Injected with Sarcoma and Normal Tissue Extracts Using Dialyzable and Non-Dialyzable Fractions of the Two Extracts as Antigens.

Extract Used for Injection into Rabbits	Extract Used for Antigen in the Precipitin Test	Gamma of Antigen Protein in the Precipitin Test						
		50 γ	20 γ	10 γ	5 γ	2 γ	1 γ	0.5 γ
Sarcoma No. 10	Sarcoma No. 10	+++G	++++G	++++G	++++G	++++G	++	±
	Dialyzed							
	Dialysate	+++++	+	0	0	0	0	0
Sarcoma No. 10	Normal Tissue			0	0	0	0	0
	Dialyzed							
	Dialysate	0	0	0	0	0	0	0
Normal Tissue	Normal Tissue			0	0	0	0	0
	Dialyzed							
	Dialysate	±	0	0	0	0	0	0
Normal Tissue	Sarcoma No. 10	0	0	0	0	0	0	0
	Dialyzed							
	Dialysate	++++	+	0	0	0	0	0

tissue extracts. The lymphoma extract contained two-thirds as much as the sarcoma extract. This amount of non-dialyzable nitrogen was extremely small and did not account for the increase in free amino nitrogen found after hydrolysis of the extracts (28), which indicated that an appreciable amount of dialyzable peptide must be present as well as the protein and non-dialyzable peptide. Thus, the increase in amino nitrogen represented about 15-16 per cent of the "net nitrogen" (total nitrogen minus ammonia, urea and nucleic acid nitrogen). Based on these studies the comparable "net nitrogens" for the extracts used in the present work were calculated, and it was estimated that the sarcoma contained 5.8 per cent of the "net nitrogen" as protein nitrogen as contrasted with 0.6 per cent in the normal tissue extract, while 10.2 per cent of the "net nitrogen" in the sarcoma extract and 15.4 per cent in the normal tissue extract existed in the form of dialyzable peptide nitrogen. The amino nitrogen determinations were not carried out on the lymphoma extract.

Interesting differences were found in the electrophoretic composition of the non-dialyzable or protein fractions from these three extracts, which were emphasized when the paper electrophoresis strips were stained with two different reagents, Bio-blue and ninhydrin. Some of the components were found to stain with ninhydrin but gave no color with Bio-blue. The protein fraction from the sarcoma extract contained three components all of which stained with both Bio-blue ninhydrin, while that from the lymphoma extract showed one component which stained with both reagents and two additional ones which gave a color only with ninhydrin. The fraction from the normal tissue extract gave no color with Bio-blue, but showed one dense and three very faint components when stained with ninhydrin. This dense ninhydrin staining component, similar to one found in the lymphoma extract, appeared to have a positive electrophoretic mobility or else was carried backward by electroosmotic flow. It is possible that this component corresponds to a breakdown product of one of the normal muscle proteins, the properties of which have been studied in great detail (1, 24). Moreover, they were reported to be easily denatured on extraction (5).

The components which stained weakly with ninhydrin but not with Bio-blue may occur in a concentration too low to be visible with Bio-blue, since this staining method is not as sensitive as the ninhydrin method. However, the fact that the components found in the

lymphoma and normal tissue extracts which stained densely with ninhydrin did not stain with Bio-blue, indicates that they were very small protein molecules or polypeptides, but of sufficient size as not to dialyze through the collodion membrane. This was further emphasized by quantitative determinations of the ninhydrin reacting capacities of these fractions, which indicated that, compared with albumin, the normal tissue fraction was more degraded than that of either the lymphoma or the sarcoma extracts.

These results are of particular interest in view of the antigenic properties of the extracts. Precipitating antibodies were formed when the lymphoma and sarcoma extracts were injected into rabbits, but not when the normal tissue extract was injected. That these antibodies were directed against the proteins in the tumor extracts was demonstrated by the fact that precipitates were obtained when the non-dialyzable material was used as an antigen, as well as when the whole extracts were used. All three proteins separated by starch block electrophoresis of the sarcoma extract gave precipitates with antisera to the whole extract.

On the other hand the substance in the tumor extracts which gave a precipitate with normal rabbit serum was found to be in the dialyzable fraction. No attempt has so far been made to characterize this substance further, since the precipitates obtained with it were readily distinguishable from those obtained in the rabbit sera after immunization with the tumor extracts. Other workers (7, 8, 14, 15, 16, 17, 18) have studied extracts of normal and neoplastic tissues by the precipitin technique, and antibodies in normal rabbit sera which react with tumor antigens have been described (6, 9, 18). However, in most of these studies cross reactions were found with antigens in normal tissues, and specificity was demonstrated only by removal of the antibodies to the normal tissues by various techniques. It appears then, that the alcoholic extracts used in this study, to a large extent, may have selected proteins peculiar to the tumor tissue and different from those extracted from the normal tissue.

In our studies cross precipitin reactions were found between the sarcoma and lymphoma proteins, indicating that these tumor tissues contain a common or related protein component. This was also suggested by paper electrophoresis experiments. It is interesting in this connection that Witebsky and Morelli (37), using the complement fixation technique, found cross reactions between alcoholic extracts

of human sarcoma and carcinoma tissues. Mann and Welker (17) also found cross reactions between various human tumors.

Not all of the rabbits injected with the tumor extracts responded well with the formation of antibodies. This may be due to the variability in animals or to the formation of incomplete antibodies, since it is well known that some rabbits are poor antibody formers. It might be argued, then, that this was the case with the normal tissue extract. However, in view of the results obtained by electrophoresis and quantitative ninhydrin determinations, the most probable explaration appears to be that the major component in this fraction is a polypeptide or small protein with a molecular weight too low for antigenic capacity as was found to be the case with some of the tuberculin proteins (27). Other reasons for lack of antigenicity of proteins are discussed by Landsteiner (12).

In all comparative studies on tumor and normal tissues the selection of the proper normal tissue for a control is of great importance. In the studies reported here rats of highly inbred strains and tumors originating in them were used, and normal muscle was chosen as the control tissue because it is the tissue in which the tumor grew, and because it contained connective tissue cells from which sarcoma originates. The importance of using this tissue as a control was demonstrated by finding a component like that of the normal muscle tissue protein fraction in the lymphoma extract. This was due, no doubt, to the incorporation of normal muscle tissue in the lymphoma extract, since the lymphoma was separated with difficulty from the surrounding normal tissue. In contrast, the sarcoma grows as a well defined, easily excised tumor, and this component was absent in the sarcoma extract. Without the normal muscle tissue control, this component might have been mistaken for a protein distinctive to lymphoma tissue.

Summary

Alcoholic extracts of a rat sarcoma were found to contain six times as much non-dialyzable or protein nitrogen as extracts prepared similarly from normal rat muscle. A lymphoma extract contained only two-thirds as much as the sarcoma extract. Both paper electrophoresis experiments and immunological reactions with sera of rabbits injected with these extracts showed that the protein fractions of the tumor and normal tissue extracts were different. The sarcoma extract

contained three electrophoretically distinct components, all of which were found to react with antisera to the extract. The lymphoma extract, on the other hand, was found to have one electrophoretic component in common with the normal tissue extract, but also two components similar to those found in the sarcoma extract.

The results indicate that the proteins in the sarcoma and lymphoma extract possessed antigenic properties. The lymphoma and sarcoma antisera showed cross reactions with their respective anigens. The production of antibodies to the tumor extracts in rabbits was variable. The extract of the normal muscle tissue did not react in the precipitin test with antisera to either tumor extract, and it did not produce antibodies when injected into rabbits. Evidence has been presented that this lack of antigenicity of the normal tissue extract may be due to the fact that the material in the non-dialyzable fraction of this extract was chiefly a small degraded protein or a large non-dialyzable polypeptide.

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THE PRENATAL GROWTH OF THE TESTES IN THE DOG

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The histology, histochemistry and hormonal secretions of the sex glands have received much attention in recent years, but there are relatively few studies on the size of the entire gonads in man or in experimental animals, and especially is this true for the fetal growth of the gonads.

The growth in weight of the human testes during the fetal period has been reported by Jackson ('09) and Wald and Scammon ('32) and the linear dimensions, by Wyndham ('43). The growth of the testes in the fetal period has been reported for the horse (Cole et al., '33), the cat (Latimer, '39), the chicken (Venzke, '54) and for the rhesus monkey in the late fetal and postnatal life (van Wagenen and Simpson, '54).

No attempt will be made to review the slightly more numerous studies on the postnatal growth of the testes, and statistical studies on the weights of the testes in adult animals. So far as is known, the following weights comprise the only quantitative data on the growth of the testes of the dog, in either the prenatal or postnatal periods.

MATERIALS AND METHODS

The growth in weight of many of the organs from this series of fetal and newborn dogs has been reported, but the data on weights of the testes collected at the time all of the organs were removed, weighed and recorded, have not been studied hitherto. The manner in which these fetuses were collected, the number of litters and the methods used in the dissections and in studying these weights, have been included in earlier reports (Latimer and Corder, '48 and Latimer, '50). There were 140 specimens in the first report but 71 additional fetuses have been added, so that the present series consists of 187 fetuses and 24 newborn puppies. In this series of 211 specimens, there are 108 males, both fetal and newborn, and 4 specimens so

small that the sex could not be determined by gross examination. The newborn testes and these very young gonads are included, with the newborn testes indicated by special symbols in the figures. The epididymides were not weighed with the testes. The weight of the testes, as used in this report, is the weight of the right and left testes weighed together.

The mother dogs were the usual heterogeneous collection of mongrels which had been gathered for use in the Physiology Department. The fetuses ranged in body weight from 3.04 to 493.5 grams and from 55 to 257 mm in nose anus length. All of the measurements were made after at least 3 months preservation in a 10% solution of formaldehyde.

Growth in Weight and Percentage Weight

The weights of the testes in milligrams, plotted on body weight in grams are represented by the heavier line in figure 1. The circles represent the weights of each pair of fetal testes and the double circles, the testes of the newborn puppies. Two straight lines are used in preference to a curved line, with a much more complex formula. These lines are drawn from the following empirical formulae: from 5 to 23 grams of body weight,

$$\text{Testes W.} = 0.26(\text{B.W.}) - 0.588, \text{ and from 23 to 440 grams,}$$

$$\text{Testes W.} = 0.076(\text{B.W.}) + 3.62.$$

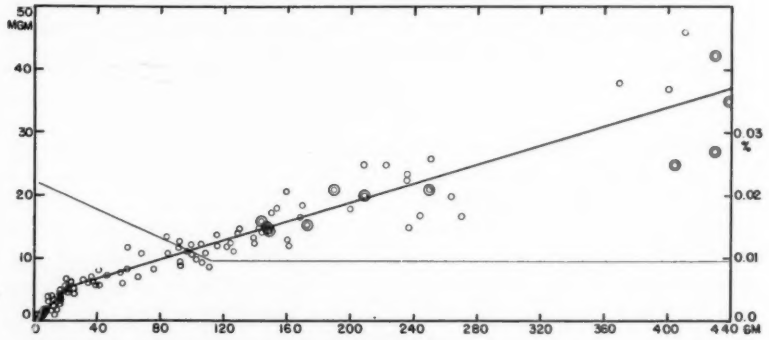

FIGURE 1

Growth of the testes in milligrams plotted on body weight in grams. Each circle represents the weight of the two fetal testes and the double circles, the testes of the newborn puppies. The lighter line without any cases, represents the testes as percentages of body weight. The percentage values are given at the right. The formulae for all of these curves are given in the text.

The average percentage deviations of the calculated from the observed values are 9.40 and 3.98% respectively, or an average of 5.00% for all of the deviations.

The lighter line without any cases shown, represents the weights of the pairs of testes as percentages of body weight. The individual percentages were plotted on a preliminary graph and there was a good deal of variation in the percentages up to 112 grams of body weight, but from 112 to 440 grams of body weight these percentage weights were more closely grouped around the regression line than the weights in milligrams shown in this figure. The first part of the line, decreasing from 5 to 112 grams of body weight is drawn from the formula:

$$\text{Percentage W.} = 0.0218\text{-}0.000108(\text{B.W.}).$$

From 112 to 440 grams of body weight these percentages average 0.0097% of the body weight. The average percentage deviations of the calculated from the observed percentages are 6.01 for the first line and 3.94% for the horizontal line, or a total average of 5.11%. These average percentage deviations show that these weights in milligrams or as percentages of body weight follow the regression lines more closely in the larger specimens, with the most variability in the very smallest specimens.

The weights of the testes plotted on body length in millimeters, in figure 2, result in a more uniform distribution of the testes weights throughout the entire range of body length than in figure 1, where the weights are crowded around the beginning of the curve. From the

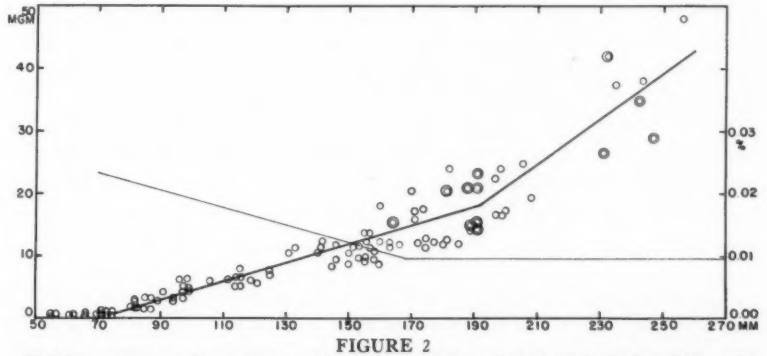

FIGURE 2

Weights and percentage weights of the pairs of testes plotted on body length in millimeters. Symbols, the same as in figure 1. The formulae are given in the text.

difference in the distribution of the same gland weights in figures 1 and 2, we may conclude that the fetuses increase in length more rapidly than in weight in the early part of the fetal period. Body weights plotted on body lengths confirm this relatively slower initial increase in body weight compared to body length (this graph is not shown).

The smallest glands appear to increase in weight very little from 55 to 70 mm of body length and no formula was obtained for this period, but from 70 to 192 mm of body length, the line representing the ponderal growth of the testes in milligrams is drawn from the formula:

Testes W. = 0.15(B.L.) –10.41, and from 192 to 260 mm of body length,

Testes W. = 0.36(B.L.) –50.73.

The average percentage deviations of the calculated from the observed values are 6.83 and 3.58% respectively, with an average of 5.20% for all of the deviations.

The lighter line, decreasing from a maximum of 0.022%, and the horizontal line represent the weights of the testes as percentages of body weight. The first part of this curve, from 70 to 167 mm of body length, is drawn from the empirical formula:

$$\text{Percentage W.} = 0.0330 - 0.00014(\text{B.L.}).$$

From 167 to 260 mm of body length the percentages average 0.0097%. The average percentage deviations for these curves are 9.90 and 8.16% respectively, with an average of 9.03% for all of the cases. The individual cases are not shown in this figure, but plotted on a preliminary graph they were rather widely scattered at first and then more closely grouped around the horizontal part of the line. Thus the percentage weights of the testes for the smallest fetuses are quite variable when plotted on body length as well as on body weight, and for both systems of abscissae, the cases are more closely grouped around the regression lines in the larger specimens.

Discussion

The prenatal growth of the dog testes is much like the growth of the human testes as described by Wald and Scammon ('32). Both human and dog testes increase as an arithmetical function of body weight, but in the dog there is a very brief period of more rapid growth at the

beginning of the fetal period, and this is not shown for the growth of the human testes. When plotted on body length, both the human and the dog testes increase more rapidly toward the end of the fetal period.

The ponderal increase in the fetal cat testes plotted on body weight (Latimer, '39) is almost identical with that of the dog, including the initial more rapid growth. However, when these weights are plotted on body length, the testes of the fetal cats are represented by a single straight line. Many of the testes of the newborn cats are far above the line, or are much heavier than the fetal testes (Latimer, '39, fig. 2), while the testes of the newborn dogs are well grouped around their regression line.

The growth in weight of the testes of the fetal horse (Cole et al., '33, fig. 1) is unique in that the maximum weight in grams is attained at about half of the full term crown-rump length, and thereafter, although the cases are widely scattered, there is a general decrease in the weights of the testes throughout the last part of the period.

Wyndham ('43) has tabulated the linear dimensions of the human testes with reference to crown-rump length and Venzke ('54) lists the linear measurements of the chicken testes according to age, and both sets of data, when plotted on their respective abscissae, show a linear increase, although the measurements are rather widely scattered. The one trait common to all of these measurements of the testes, of whatever species, is their marked variability.

The testes of the fetal dogs have their maximum percentage of body weight (0.022%) at the beginning of the fetal period, and then decrease to an average of 0.0097%. The length of this horizontal line, representing this terminal average percentage weight, varies in figures 1 and 2, due to the type of abscissa used. The use of body weights as abscissae causes a concentration of the relative weights of the testes in the early part of the fetal period, resulting in a longer horizontal percentage line in figure 1 compared to that in figure 2 in which these same percentage weights are plotted on body length.

The cat testes as percentages of body weight (Latimer, '39) have their maximum of 0.11% at the beginning of the fetal period, followed by a decrease to 0.013% at the end of the period. Thus the cat testes are 5 times as heavy as the dog testes at the beginning, but at the end of the fetal growth their percentage weights are more alike.

The human testes as percentages of body weight (Jackson, '09) decrease from their initial maximum, but they are relatively heavier throughout the entire period than the testes of the dog or cat.

It is interesting to note that while the testes are increasing 120 times in total weight, or from 0.4 to 48 milligrams, the body weight is increasing 162 times, and this difference explains the decrease in percentage weight of the testes from 0.022 to 0.0097%. The body length, a linear dimension, increases but 4.7 times in the same period.

In general, we may conclude that the prenatal growth of the dog testes, whether measured in milligrams or as percentages of body weight, resembles the similar growth of the testes in man and in the cat. Furthermore, their marked variability is like that of all known data on the weights of the fetal testes.

Summary

The rate of increase in weight of the fetal dog testes plotted on body weight is most rapid in the early fetal period (5 to 23 gm of body weight) and thereafter the rate of increase is less rapid. The growth in both of these periods may be represented by straight lines.

The same weights plotted on body length, increase most rapidly in the last third of the fetal period, and again this growth is represented by two straight lines, but the second rises more rapidly.

The pairs of testes at the beginning of the fetal period form an average of 0.022% of the body weight and this decreases to an average of 0.0097% in the latter part of the period.

Empirical formulae are given whereby the weights in milligrams or the percentage weights may be estimated from either body weight in grams or body length in millimeters.

The total increase in weight of the testes during the fetal growth period is 120 times, and the total increase in body weight is 162 times. The body length, which of course is a linear dimension, increases but 4.7 times.

The weights and percentage weights are all rather variable and the percentage weights are most variable in the smallest fetuses.

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STUDIES ON THE TU^{W} STRAIN OF *DROSOPHILA MELANOGASTER*

I. PHENOTYPIC AND GENOTYPIC CHARACTERIZATION[1, 2]

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INTRODUCTION

Our information on the subject of pseudotumors in *Drosophila* is built up of bits and pieces of information for various strains of independent origin. Interpretations of data are often conflicting, and since it is not known whether all strains showing pseudotumors do so because of the same underlying sequence of reactions, generalization is difficult.

The term pseudotumor (Barigozzi, Castiglioni, and DiPasquale, '53) is used to refer to blackened aggregations of cells found in various strains of certain *Drosophila* species. The pioneer study in this field was published in 1918 by Dr. Mary B. Stark. Today we still know little of the morphology of the cells which initiate pseudotumor histogenesis and equally little about the constituents of the haemocyte population of *Drosophila melanogaster* larvae. Fixed material gives a distorted picture of the morphology and behavior of some haemocytes (Rizki, '53), and consequently future studies utilizing the phase microscope seem in order. At present, however, it is impossible to be sure that the cells which aggregate and form the core of pseudotumors are actually blood cells as Oftedal ('53) has concluded.

The black pigment which subsequently forms in pseudotumors is probably melanin (Hartung and Tillinghast, '49). Melanotic pseudotumors grow (increase in mass) only during larval stages.

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[2] A part of this study was completed at the Biology Department of the Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.
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Mitoses are rarely, if ever, seen in pseudotumors. Although there are strains in which larvae develop pseudotumors in the first and second instar, these black masses usually appear in the third instar and persist throughout the rest of the life of the fly. The pseudotumors are usually benign, and if lethal effects are observed, these are generally independent of the melanotic masses. This is not to say, however, that lethality and pseudotumors do not have the same underlying cause.

The incidence of melanized pseudotumors varies greatly from strain to strain, and within a strain the incidence may be increased or decreased by treatment with ionizing radiations, chemicals, and by modification of the culture conditions with respect to nutrition and temperature. The fact that the production of pseudotumors in various species of *Drosophila* is inherited is well established. The condition may be due to one or more "major" genes, and a given strain may contain in addition genes suppressing or enhancing "tumor" incidence. Within a given strain pseudotumor incidence may be different between the two sexes.

The Wellesley pseudotumorous strain, tu^{w}, described in this study originated some twenty years ago (November, 1934) from "tumor" bearing flies found by Dr. John Gowen in the offspring of Swedish-B wild type *Drosophila melanogaster* fed for a 2 year period upon medium containing dibenzanthracene at an almost lethal concentration. Before abandoning work on this strain Gowen showed that the tumor phenotype was caused by at least two genes located upon chromosome II. The strain was obtained from Dr. Gowen and has been maintained under constant selection (L.P.W.) for pseudotumors at Wellesley since 1943. The studies of the phenotype of the stock were started at Wellesley (L.P.W. and J.L.L.) and continued at Brookhaven National Laboratory (L.P.W.). The studies on the inheritance of the pseudotumors were done at Brookhaven (R.C.K.). All experiments were carried out at 25° C.

The Phenotype of the Pseudotumorous Strain

1) *Description of the Pseudotumors*

The strain is characterized by a high incidence of pseudotumors, located on the surface or embedded in the fat body of the larva, and persisting throughout the life of the adult (Figure 1, a,b,c). It has been shown that if the developmental period is lengthened by grow-

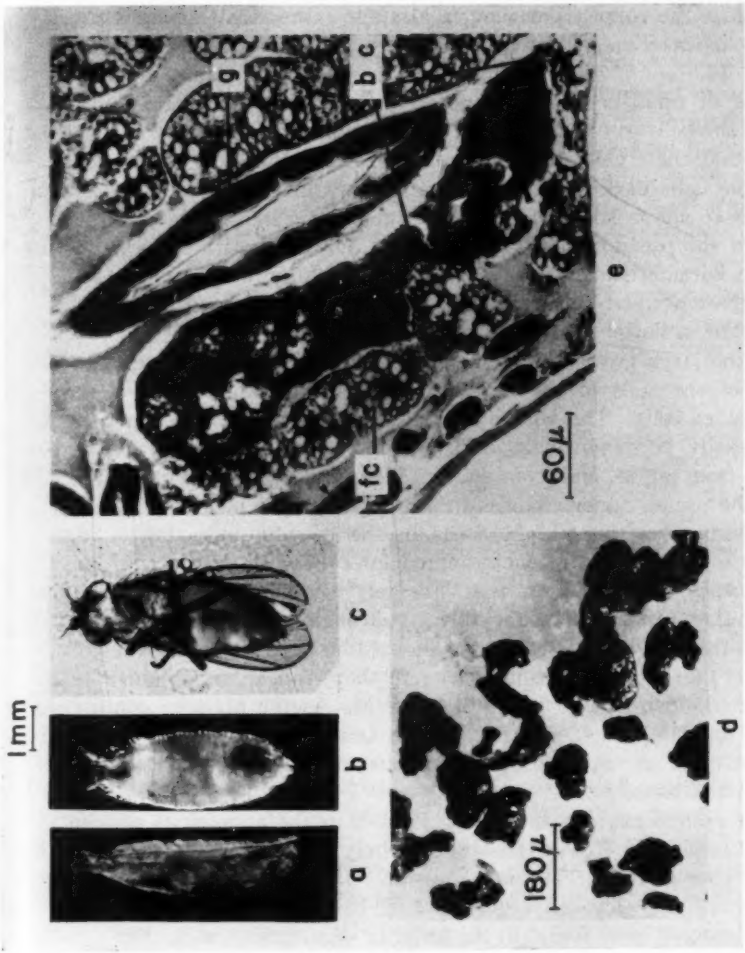


FIGURE 1

a) Pseudotumors in late larva, b) pupa and c) adult female *Drosophila melanogaster*. d) Excised pseudotumors from adults. e) Section through a pseudotumor of a larva, 120 hours after hatching, stained by a modified Bodian protargol method. g—gut; bc = tumor ("blood") cells; fc—fat cells.

ing larvae from this strain on a medium containing dinitrophenol or high concentrations of arginine (Wilson, '47, '49) the pseudotumors appear at the correct developmental stage rather than at the correct chronological time. Normally the pseudotumor becomes visible at 97 to 102 hours after hatching, as deposits of melanin appear in masses of small cells (of unknown origin) along the margin or between the cells of the fat body. It is possible in the light of Oftedal's work that these cells are blood cells. However, we have no evidence that the cells darken prior to their aggregation as Oftedal suggests. Figure 1e shows some of these cells along the amorphous melanized area of the pseudotumor. The cells are polygonal with spherical nuclei. Characteristically, the cytoplasm is faintly granular but some cells show perfectly clear cytoplasm. Those cells located between fat cells show a flattened, spindle shape.

As the larva grows the melanized area increases in size, surrounds some of the cells of the fat body and cuts them off from contact with other cells. The whole pseudotumor becomes encapsulated and may easily be removed as a discrete irregular mass from the late larva, from pupae, and from adults (Figure 1d). There is no evidence that the pseudotumor changes in size or degree of melanization after puparium formation. Light areas in the pseudotumors taken from adults in Figure 1d are encapsulated fat cells with only a thin layer of melanin over their surface. The fatty tissue within the pseudotumor therefore remains uncytolyzed throughout pupation.

The melanin is first seen as small dot-like deposits within the cells, but as the deposits become heavier, they appear to organize into elongated, fusiform, or string-like masses. As the pigment continues to accumulate, the cellular structure becomes obliterated, and the pseudotumor appears as an amorphous, friable, black mass by the end of the larval period.

The pseudotumor is invariably located in the abdominal segments of the larva. Of 104 larvae observed only six failed to have pseudotumor sites in the larval tenth segment. Of these 76 also had pseudotumors in the eleventh segment, and 10 in the ninth. Occasional pseudotumors were found in the anterior segments as well. The number of sites varied from one to six and averaged 2.2 sites per larva. In the adult the pseudotumor has been observed only in the abdomen, more often on the ventral than the dorsal side, and suspended in the haemolymph or loosely attached to the abdominal organs. The aver-

age number of sites in the adult male is 2.2 and for the female is 2.0. Most pseudotumors are less than one segment in length, but occasionally a single mass occupies two segments.

2) *The Effect of Nutrition on the Incidence of Pseudotumors*

That quality and amount of food effect the incidence of melanized pseudotumors has been established by a number of investigators. The incidence of pseudotumors in flies may be increased if the medium upon which they are grown is supplemented by B vitamins. Friedman ('55) has most recently discussed this literature. The tu^w stock is also sensitive to a changed nutritional environment. In optimally-fed, fresh cultures almost 100% of the flies have one or more pseudotumors. In poorly fed or old cultures the incidence of pseudotumor-bearing flies rapidly decreases. Table 1 shows the results of

TABLE 1
Incidence of Pseudotumors in a Culture of Flies Raised on Cornmeal-Molasses Medium.

Period of Emergence (days)	No. of Flies		% Pseudotumors	
	Males	Females	Males	Females
1-3	113	101	100	100
3-6	96	99	99	95
6-10	85	92	97	98
10-20	156	202	36	11

a count of tumor-bearing flies reared on regular cornmeal-molasses medium seeded with live baker's yeast. Where incidence of the melanized pseudotumors was reduced in old cultures, males show a higher frequency of pseudotumors than females.

A more accurate measure of the effect of food quantity and quality on incidence of melanized pseudotumors may be obtained by using sterile media with known concentrations of dead yeast. A series of experiments was set up in which sterile eggs and sterile medium were prepared as described by Wilson and Birch ('44). Harris' dried brewers' yeast was added in varying concentrations to the sterile media. Eggs were collected over a two hour laying period, sterilized, washed, and added to 30 ml. of the medium in 3.5 ounce bottles (approximately 100 eggs to each bottle). Table 2 shows the results of counts made on flies which underwent development on various concentrations of yeast. A second series of experiments (cf. Table 3) using Anheuser Busch Strain G brewers' yeast gave similar results except that a lower concentration of yeast was required to raise

pseudotumor incidence to 100%. The sexes were not recorded separately in this case. It is evident that some substance necessary for development of melanized pseudotumors in the tu^w strain of flies was lacking or in low concentration in the Harris' yeast. A short time

TABLE 2
Incidence of Pseudotumors (Sterile Medium with Harris' Brewers' Yeast)

Concentration of Yeast (g) per 100 ml. Food	Number Cultures	Total Number of Flies		% Pseudotumors	
		Male	Female	Male	Female
16	4	84	96	94	94
8	6	168	143	83	85
4	24	676	723	47	43
2	6	185	154	66	54
1	8	198	202	26	15

TABLE 3
Incidence of Pseudotumors (Sterile Medium with Brewers' Yeast, Strain G).

Concentration of Yeast (g) per 100 ml. food	Number Cultures	Total Number of Flies	% Pseudotumors
8	4	214	100
4	12	727	98
3	12	531	70
1	8	364	21
0.75	4	127	14

after these experiments were performed the Harris' yeast failed to support growth at all in sterile cultures. This brewers' yeast was grown by Harris' Laboratories of Tuckahoe, New York, and obtained from the Massachusetts General Hospital, Boston. We learned later that this yeast had been stored for a long time in a hot room which might account for the fact that it would not support growth.

From the above experiments it is evident that amount and quality of food are important variables with respect to pseudotumor incidence. Both aggregation and melanization phases of pseudotumor production may be interrelated in a very complex manner. Thus by varying a single physical or chemical parameter, one may change larval physiology in an enormous number of ways. A changed larval nutrition, for example, may produce a modified rate of growth, a changed series of endocrinological interactions between the brain and the components of the ring gland, a changed pH and oxidation-reduction potential of the haemolymph and a modification of its content

of vitamins, amino acids and minerals, as well as of its cellular population. Such changes may affect both aggregation and melanization even if both processes are totally independent.

3) *Viability of Various Developmental Stages*

The wild type strain generally used for comparison throughout the paper is the Swedish-B. The stock came originally from the late Dr. C. B. Bridges and has been inbred continuously at Wellesley since 1938. The Swedish-B and the pseudotumorous stock have been maintained under similar environmental conditions for more than a decade. The number of adults which developed from 1,610 eggs laid by females of the pseudotumorous strain was determined. Only 56% of the eggs gave rise to adults. Data on the percent survival of wild type and pseudotumor strains of flies for various developmental stages were therefore determined (cf. Table 4). The only significant differ-

TABLE 4
Relation Between Percent Survival and Stage of Development.*

	Wild		Pseudotumor	
	No.	%	No.	%
Eggs to larvae	483	93.9	524	75.7
Larvae to pupae	400	91.5	346	87.3
Pupae to adults	182	97.8	178	92.3

* Four to six tests using different sets of parents were made in each case at intervals of days or weeks apart.

ence observed between pseudotumor and non-tumor strains is in the percent of eggs yielding larvae. Since the frequency of unfertilized eggs is not known in either case, these data do not necessarily indicate that viability during embryogenesis is different in the two strains. These data indicate that the actual development of pseudotumors has little if any effect on viability.

4) *Adult Viability*

Adult flies of the pseudotumor strain fail to live as long as wild type adults. Comparative data on longevity are shown for males and females in Figure 2. In both strains females live longer than males. Mated pseudotumorous males or females live on the average 65% as long as mated wild type males or females. The fifty percent time, T_{50}, is defined as the time at which fifty percent of the flies of a given group have died. The T_{50} for pseudotumorous males is 56%

the wild type T_{50}, while the T_{50} for pseudotumorous females is 73% of the wild type value. It has been noted that virgin males and females live longer than mated males and females in both strains (cf. Table 5).

The duration of life of pseudotumor flies is more affected by adverse conditions than that of the wild flies. To demonstrate this one

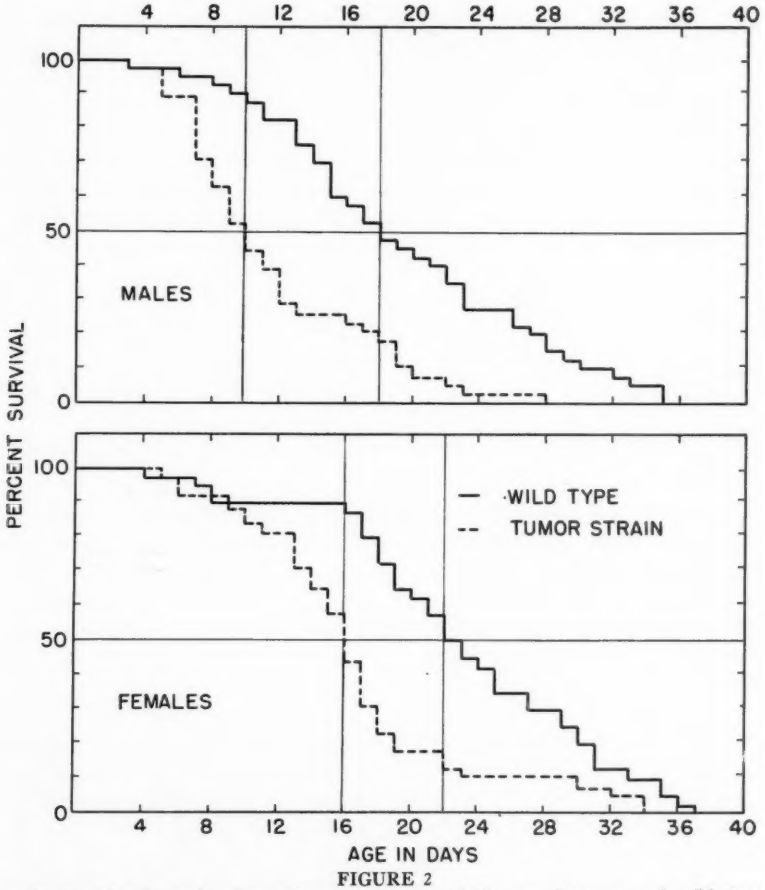


FIGURE 2

Comparative longevity for males and females of the pseudotumor and wild type strain of *Drosophila melanogaster*.

hundred males and 100 females (less than 24 hrs. old) of each stock were placed in bottles without food or water. The percentages of survivors at intervals are shown in Table 6. Again the males of both

TABLE 5
Comparative Longevity of Adults in Days.

	Wild		Pseudotumor	
	Mated	Virgin	Mated	Virgin
Average female	23.6	30.5	15.9	22.2
Oldest female	42	56	34	42
Average male	19.9	25.7*	12.8	25.3*
Oldest male	38	56*	28	49*

*Taken from another experiment.

TABLE 6
Percent Survival of Wild and Pseudotumor Flies in the Absence of Food and Water.

	Wild		Tumor	
Time in Hours	Males	Females	Males	Females
0	100	100	100	100
6	100	100	100	100
12	98	100	96	97
24	93	97	27	82
30	70	92	0	36
36	4	85	0	13
42	0	16	0	0
48	0	0	0	0
	—	—	—	—
Approximate time for 50% to die (hrs.)	32	39	20	28

pseudotumor and wild strains are less resistant to this type of stress than the females. If the data are plotted parallel, sigmoid survival curves are produced. When the approximate time required for 50% of the flies to die is calculated it is found that the values for pseudotumorous flies are approximately 63% (♂♂) and 72% (♀♀) those of wild type flies.

5) *Egg Size*

Since size and rate of growth in *Drosophila* depend upon nutritive conditions and temperature, it is especially essential for valid comparative studies involving these features that all environmental conditions be uniform for both the wild and tumor flies. As in all experiments, flies were kept at 25° C. and the same culture medium was used for both types of flies.

Males and females from uncrowded cultures were fed for five days

on freshly yeasted food and eggs collected on pyralin spoons (Palmer, '39) during a two-hour laying period. They were measured with the micrometer ocular of a Zeiss Opton stereo-microscope, and the weights calculated as described by King and Wilson ('54). The results are

TABLE 7
Size of Eggs in Pseudotumor and Wild Strains.

	No.	Average Length	Standard Deviation	Average Width	Standard Deviation	Calculated Weights*
Tumor	114	0.4745 mm.	0.0209	0.1910	0.0083	10.2 μg
Wild	112	0.5136 mm.	0.0250	0.2027	0.0112	12.5 μg

* Density = 1.13.

shown in Table 7. The standard t-test showed that the differences in length and width between the two strains are highly significant.

6) *Larval Growth Rate*

Experiments were performed (Lowry, '53) in which larvae of known ages were reared on a modified culture medium[4] containing less cornmeal than usual and no oatmeal. These omissions make a more transparent medium and thus facilitate the removal of larvae (each 6 hours). Larvae were reared in 40 mm Petri dishes to which 3 ml of medium was added. Twenty eggs were placed in each dish. Eggs were collected during a 2-hour laying period and transferred immediately to the culture dishes. Ages given refer to the median hour of egg laying plus or minus one hour.

Beginning at 20 hours, between 100 and 200 larvae were removed from the dishes at each 6-hour interval and their instar and length determined by the method of Alpatov ('29). A Bausch and Lomb compound microscope, equipped with a 10x ocular, an ocular micrometer and a 48 mm wide field objective were used in the measurements.

The resulting data are given in Table 8. When plotted the length appears to vary exponentially with time.

Since larval growth appears to be exponential, the data may be represented by an equation of the form $y = ie^{kt}$ or $\overline{Y} = a + bx$ where:

[4] Components (per liter water) 65 cc Karo white corn syrup, 19 g agar, 31 g yellow cornmeal, 6 g brewers' yeast (Anheuser Busch, Strain G).

y = larval length (mm)
t = larval age (hours)
i = the y intercept
$\overline{Y}$ = log y
a = log i
$b = k \log e = 0.43429k$ = slope of the growth curve on semi-log paper.
$x = t$
$k = 0.693/T_{½}$
$T_{½}$ = the time (hrs.) for larval length to double.

The line of best fit representing growth during each instar was determined. Each point was weighted according to the number in the sample. Starred data (cf. Table 8) were not used in these calculations since they probably represent abnormal individuals. In Figure 3 the calculated curves are presented along with the points representing the average values at each six hour collection period. The results of the analysis are shown in Table 9.

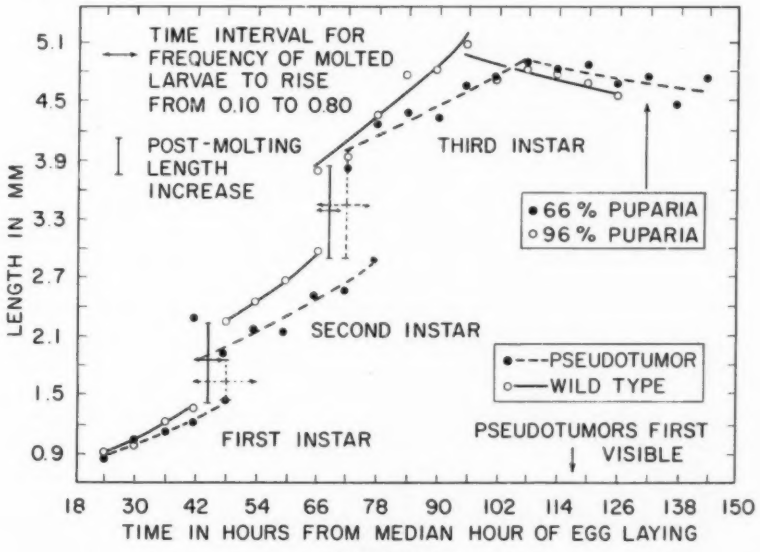


FIGURE 3
Growth curves for larvae of the pseudotumor and wild type strain of *Drosophila melanogaster*.

TABLE 8

GROWTH OF WILD TYPE (+) AND PSEUDOTUMOROUS (tu) LARVAE

Time (hrs)	1st Instar Larvae				2nd Instar Larvae				3rd Instar Larvae			
	+		tu		+		tu		+		tu	
	No.	Average Length (mm)	No.	Average Length (mm)	No.	Average Length (mm)	No.	Average Length (mm)	No.	Average Length (mm)	No.	Average Length (mm)
20	8*	0.931	10*	0.923								
24	179	0.888	199	0.847								
30	176	1.005	168	1.047								
36	194	1.205	187	1.105	24*	2.333	8*	1.990				
42	172	1.347	165	1.180	26*	2.564	34	2.287				
48	20*	1.696	50	1.427	116	2.225	60	1.948				
54	8*	1.749	11*	1.790	164	2.450	103	2.199	9*	3.921		
60	8*	1.752	4*	1.250	162	2.666	141	2.131	12*	4.139	1*	4.000
66	5*	1.799	2*	1.615	107	2.953	109	2.524	25	3.768	9*	3.905
72					8*	3.018	49	2.572	125	3.919	50	3.780
78					7*	2.933	20	2.891	155	4.352	73	4.255
84					1*	2.691	9*	2.785	182	4.777	107	4.370
90									139	4.817	102	4.319
96									179	5.079**	111	4.655
102					2*	2.615	2*	2.000	168	4.725	128	4.750
108									175	4.831	71	4.885**
114									104	4.789	62	4.837
120	Pseudotumor first visible								109	4.691	69	4.872
126									80	4.561	52	4.661
132									6*	3.435	35	4.744
138									16*	4.441	18	4.464
144											13	4.726

* not used in statistical treatment.
** beginning of shortening of third instar larvae.

TABLE 9
Comparative Growth Indices for + and tu *Drosophila* Larvae.

Instar	i (mm)		k hrs^{-1}		t_1 (hrs)		t_2 (hrs)		y_1 (mm)		y_2 (mm)		t_2-t_1 (hrs)		y_2-y_1 (mm)	
	+	tu	+	tu	+	tu	+	tu	+	tu	+	tu	+	tu	+	tu
1	0.499	0.559	0.0239	0.0188	24	24	42	48	0.885	0.877	1.361	1.375	18	24	0.476	0.498
2	1.122	1.101	0.0144	0.0122	48	42	66	78	2.237	1.835	2.899	2.845	18	36	0.662	1.010
3 lengthening	1.955	2.595	0.0101	0.0059	66	72	96	108	3.816	3.982	5.171	4.931	30	36	1.355	0.949
3 shortening	6.471	5.955	−0.0027	−0.0018	96	108	126	144	4.974	4.899	4.582	4.590	30	36	−0.392	−0.309

+ = Swedish-B wild type larvae.
tu = pseudotumorous larvae.
t_1 = age at beginning of stage.
t_2 = age at end of stage.
y_1 = length at beginning of stage.
y_2 = length at end of stage.

	⟵ + ⟶ Length increase (mm) = A	%	⟵ tu ⟶ Length increase (mm) = B	%	B/A
Instar 1	0.476	11	0.498	12	1.05
PMLI 1	0.876	20	0.460	11	0.53
Instar 2	0.662	15	1.010	25	1.53
PMLI 2	0.917	22	1.137	28	1.23
Instar 3	1.355	32	0.949	24	0.70
	4.286	100	4.054	100	

PMLI = Post-molting length increase.

One may summarize the pattern of normal growth as follows. The rate of increase in larval length with time is exponential. The *relative* growth rate is greatest for first instar larvae, intermediate for second instar larvae and least for third instar larvae. With respect to increasing rates of *actual* length increase for the three instars, the above order is reversed. Half way through the third instar the larva undergoes a comparatively slow shortening. At the time directly after a molt the increase in length is extremely rapid. This post-molting growth accounts for 40% of the total increase in length from 24-96 hours.

Larvae from the pseudotumorous strain differ markedly from wild type larvae. Differences are detectable long before the pseudotumors appear. Relative growth rates for each instar are characteristically lower than wild type rates. Standard t tests for the significance of the difference between b values for wild type and pseudotumorous larvae were made. Only the slopes for the first half of the third instar gave a significant difference ($P = 0.054$). However, a striking difference exists between the maximum length for the instars of wild type and pseudotumorous larvae. The pseudotumorous values are 1.2-2 times greater. Furthermore, pseudotumorous larvae increase in length less than do wild type larvae during the first post-molting length increase and during the third instar. The situation is reversed during the second instar and the second post-molting length increase. The two classes of larvae which result do not differ significantly in length. Therefore, although the growth pattern is quite different between the two strains, the resultant organisms differ very little with respect to length.

7) *Developmental Times*

The time between puparium formation and eclosion in the two strains of flies was noted. In a few cases exact times were recorded, and in others the exact time was determined within 15 minutes to two hours. From these data and from the experiment on growth rate of the larvae a time table for development of wild and pseudotumorous flies was obtained, and the results are shown in Table 10.

8) *Adult Growth*

In Figure 4 data are presented comparing growth (increase in mass) between wild type and pseudotumorous adult flies. Whereas males

of both strains remain constant in weight, females gain rapidly. Pseudotumorous females far surpass wild type females, and in a period of 24 days double their weight.

TABLE 10
Time Table for Larval and Pupal Development of *Drosophila melanogaster*.
(Time in hours)

Stage		Wild Type	Pseudotumor
Egg laying		0	0
Hatching		20–24	20–24
1st instar (maximum range)		20–66	20–66
10% molted		42	42
50% molted		45	48
80% molted		48	54
2nd instar (maximum range)		36–84	36–84
10% molted		66	66
50% molted		69	72
80% molted		72	78
3rd instar (maximum range)		54–138	60–144
85% formed puparia		126	144
Puparian formation to eclosion (duration in hrs)	males	97.5	107.9
Range	males	93–102	98–121
(Duration in hrs)	females	94.3	103.4
Range	females	90–102	96-116
Hatching to eclosion (duration in hrs)	males	201.5	232.4
Range	males	196-205	221-238
Hatching to eclosion (duration in hrs)	females	197.5	227.7
Range	females	192-205	218–235

9) *Fecundity*

Numerous investigators have shown that food quantity, quality and population density are factors modifying egg production by *Drosophila melanogaster*. This literature has been reviewed by Sang ('50). Since these and other environmental conditions may modify different strains to a different degree, it is necessary to make all comparisons under conditions as nearly identical as possible.

Well-fed females reared at 25° C. were used for all experiments. Eggs were collected on pyralin spoons and counted under a binocular microscope. Duplicate counts were made in all cases. In the first experiment young females were fed for five days on well yeasted corn-

meal-molasses medium, in order to assure optimum production and little retention of eggs. The unetherized flies (approximately 25 males and 25 females) were transferred to empty half pint bottles and eggs were collected for one hour on spoons coated with 1.5% agar and

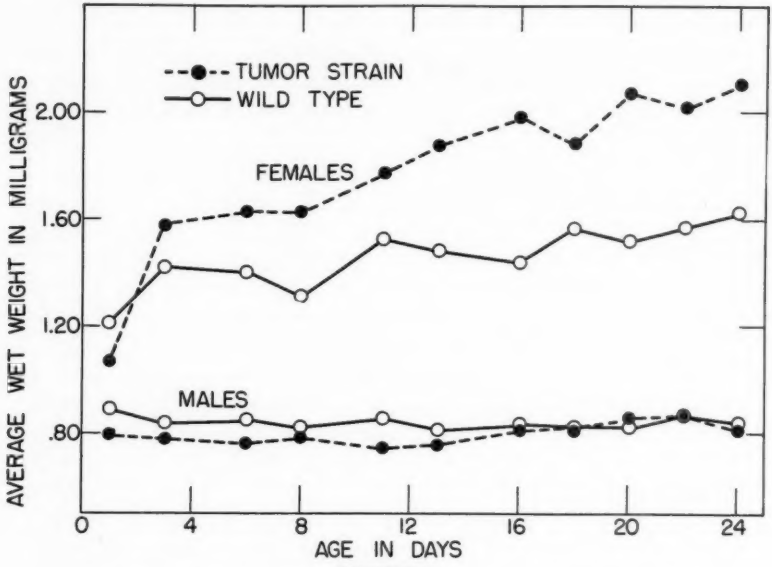


FIGURE 4
Increase in mass with age for adult wild type and pseudotumor flies.

yeasted with live baker's yeast. At the end of the period the spoons were removed, the eggs counted, and the flies etherized and counted. The results are shown in Table 11. Mated wild females lay 3.9 times as many eggs per hour as do the pseudotumorous flies.

To find whether this difference is a consistent one throughout the life of the flies, total egg production was determined for 40 pairs of

TABLE 11
Comparative Egg Production by Mated Female Flies*

	No. females	No. bottles	No. eggs	Average No. Eggs/hr/♀
Wild	258	25	1244	4.82
Pseudotumor	391	30	488	1.25

* Fed live baker's yeast.

flies by collecting eggs every day on spoons coated with regular culture medium and well yeasted with live baker's yeast. Spoons were changed each day and new males added, if the old ones had died. In addition to mated pseudotumor and wild type flies, data were collected on the total number of eggs laid by virgins and by mated females from the pseudotumor strain which showed no visible pseudotumors. A summary of the results is shown in Table 12. The data are not complete for the "pseudotumor" series, since these flies were dissected before death, but after the end of the egg-laying period to determine that "tumor" sites were completely absent. Therefore data

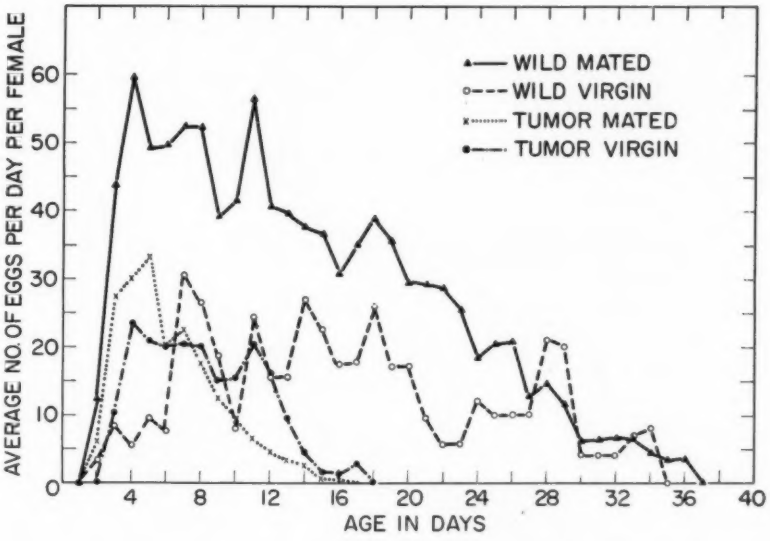


FIGURE 5

Daily egg production as a function of age for pseudotumor and wild type *Drosophila melanogaster* females.

are not available on the life span of these flies. Figure 5 shows the average daily egg production per female as a function of time for each of the categories. Figure 6 shows average total egg production as a function of time for each of the categories.

From these data a number of conclusions with regard to the comparative fecundity of the two strains may be drawn. Phenotypically

TABLE 12
Comparative Data for Fecundity of Wild and Pseudotumorous Strains of *Drosophila melanogaster*

	A Wild Type Mated	B Pseudo-tumor Mated	C "Pseudo-tumor" Mated*	D Pseudo-tumor Virgins	E Wild Type Virgins	Relative Fecundity Indices					
						B/A	C/A	D/E	E/A	D/B	B/C
Average lifetime egg production/female	810	177	284	210	473	0.22	0.35	0.44	0.58	1.19	0.62
Average egg production/female/day	37	12		14	16	0.32		0.82	0.43	1.17	
Average days of egg production	21	11	13	10	26	0.52	0.62	0.38	1.24	0.91	0.85
Average egg production/female/laying day	39	16	22	21	18	0.41	0.56	1.17	0.46	1.31	0.73
Maximum lifetime egg production/female	1369	330	383	315	655	0.24	0.28	0.48	0.48	0.95	0.86
Maximum egg production/female/day	80	66	57	24	35	0.82	0.71	0.68	0.44	0.36	1.16
Day of above maximum egg production	6	4	3	2	17						

* Females from pseudotumor strain showing no visible pseudotumors.

wild type pseudotumorous females are significantly more fecund than phenotypically pseudotumorous females. However, the females from the pseudotumor strain showing no melanized tumors behave more like phenotypically pseudotumorous flies than like wild type flies.

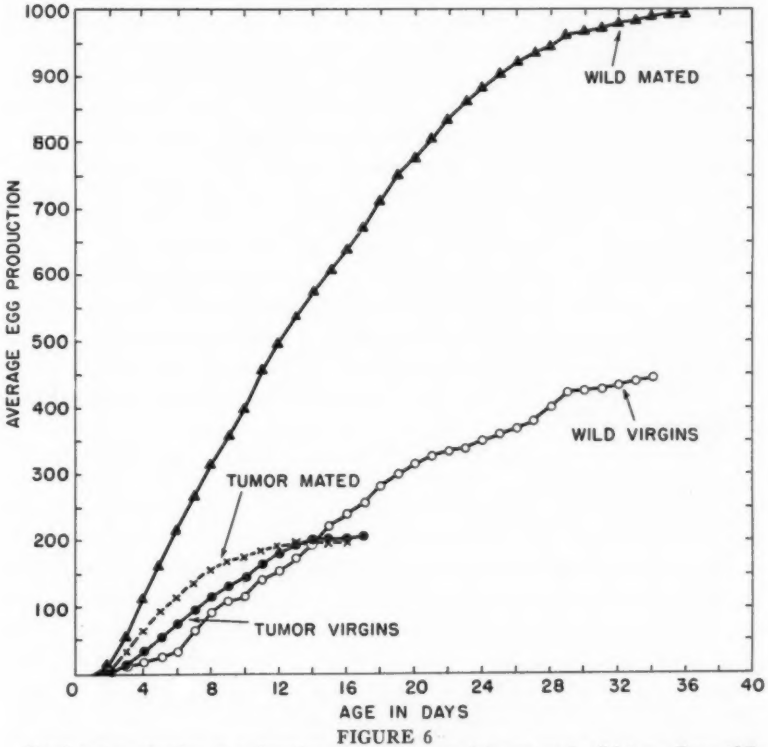


FIGURE 6

Total egg production as a function of age for pseudotumor and wild type *Drosophila melanogaster* females.

Therefore the lowered fecundity of pseudotumorous flies is not due to the presence of pseudotumors *per se*.

Wild type virgins are less fecund than mated wild type females, but this is not true for the pseudotumorous strain. It is a well known fact that the stimuli attending copulation induce egg laying by female *Drosophila melanogaster*. This appears not to be the case for the

pseudotumorous strain. Pseudotumorous virgins, however, are still less fecund than wild type virgin female flies.

The normal relation between daily egg production and time for mated wild type *Drosophila* females under our experimental conditions appears to be as follows. No eggs are laid for the first two days after eclosion. Egg laying commences on the third day and reaches a maximum on the fourth day. The daily rate of egg production declines exponentially at a rate such that at 20 days after eclosion the rate is one half the maximum rate. At twenty-two days the exponential rate of decline in daily oviposition becomes four times faster. Oviposition ceases at the 37th day. Mated pseudotumorous females reach a peak in oviposition (½ the control value) on the fifth day. There follows an exponential decline in daily oviposition such that the daily oviposition rate is halved each 3 days. By the fifteenth day oviposition has ceased. The relation between daily egg production and time for virgin females is represented by a ∩-shaped curve. For both wild type and pseudotumorous flies the plateau value for daily oviposition is about 1/3rd the maximum value for mated wild type female flies. The total oviposition period for wild type flies is about twice that of pseudotumorous flies.

There is, in an extensive literature (cf. Gowen and Johnson, '46 and Donald and Lamy, '36/'37), no evidence for rhythmical behavior with respect to egg laying in mated females of *Drosophila melanogaster*. However, our experiments do show an apparent periodicity in the daily egg production of virgin Swedish-B females. Four peaks of egg laying activity occur; each separated from the previous one by a period of approximately four days.

10) *Egg Retention*

Ten females of each stock were dissected to determine whether any abnormalities of the reproductive system could account for the lowered egg production of the pseudotumorous flies. The only significant difference noted was the number of mature eggs in the ovaries. Table 13 shows the averages for ten females in each case. In all cases more mature eggs were found in the ovaries of flies of the pseudotumorous strain than in those of the wild type flies. Eggs were seldom found in the uterus of the pseudotumorous strain whether virgin, mated, or pseudotumor-free, but they were found in every uterus of wild flies. The tumor flies looked swollen with eggs, and it is possible to account

for the difference in weight between females of the two strains (cf. Figure 4) wholly by the accumulation of eggs in the pseudotumor female.

Finally, the flies with no melanized tumor from the pseudotumor strain lay only 35% as many eggs as the wild type, their period of

TABLE 13
A Comparison of the Retention of Mature Eggs in Ovaries (Average of 10 Flies).

	Pseudotumor strain		Wild type strain	
	Age in days	No. eggs	Age in days	No. eggs
Mated	10	63.1	10	28.9
	20	91.1	21	24.0
Virgin	28	92.0	28	40.8
tu^W genotype + phenotype	28	101.9		

production is only 60% as long, their patttern of egg laying is similar to those flies showing pseudotumors, and they retain eggs as do the pseudotumor-bearing flies. It therefore appears that the possession of melanized pseudotumors has no effect on egg laying behavior.[5] Females of this strain of flies can metabolize eggs, but fail to lay more than a fraction of these eggs. The copulatory stimulus which elicits egg laying behavior in most *Drosophila* strains has little or no effect.

The abnormalities demonstrated for the tu^W strain of flies include the production of smaller eggs, lowered yields of larvae from the eggs laid, lowered longevity of adults, lowered resistance of adult flies to starvation and desiccation, an abnormal pattern of larval growth, the failure by females to lay formed eggs, and the presence of pseudotumors. Such abnormalities would appear to be manifestations of a fundamental metabolic disturbance of the organism as a whole. Artificial selection for "tumors" appears to have fixed these other characters in the strain at the same time, and one wonders if the genes responsible for the pseudotumor phenotype affect the other characteristics as well.

[5] Perhaps during the larval stage the presence of pseudotumor cell aggregates, whether melanized or not, somehow damages the anlage of the reproductive ducts. A subtle difference between wild type and pseudotumor oviducts might be demonstrated in sectioned material.

11) *The Hereditary Control of Pseudotumor Incidence*

METHODS AND RESULTS

Males of the pseudotumor strain have a pseudotumor incidence of 100%; females 98%. If reciprocal crosses are made between flies of the pseudotumorous stock and flies of the Canton S wild type stock, the tumor incidence in F_1 flies is less than 1/1000 (8/8,595). The character therefore behaves as a recessive.

To study the interactions of the X, second and third chromosomes the following cross was made. P_1 tu^w female X y^{S1} sc^8 InS y^{3P}; dp b Pm^1/al^2 In (2R) Cy lt^3 sp^2; ru h D Ins CXF ca/Sb In (3R) male. The male in question was from a non-pseudotumor stock synthesized by Professor H. J. Muller ('42) in which the X, second and third chromosomes contain inversions and dominant and recessive marker genes. The intact, non-pseudotumor chromosomes may be followed through further crosses, since most crossover products are not recovered. By suitable crosses between F_1 individual stocks were produced which were free of pseudotumor 2nd chromosomes (Pm/Cy), 3rd chromosomes (Sb/D) or X and third chromosomes (y,Sb/D). Subsequent crosses between Sb/D males and tu females gave flies heterozygous for Sb and D. Crosses between y, Sb/D females and tu^w males gave other necessary classes: y/tu^w; Sb/tu^w, y/tu^w; D/tu^w, y/Y; Sb/tu^w, and y/Y; D/tu^w. Data for the incidence of pseudotumor-free flies for each genotype are presented in Table 14.

The data clearly show that substitution of both tu^w second chromosomes reduces pseudotumor incidence to zero. Data from other experiments prove that substitution of one tu^w second chromosome by a second chromosome from a non-pseudotumor strain also reduces pseudotumor incidence to zero no matter what the X or III chromosomal constitution. Thus in confirmation of Gowen's original conclusions it was shown that one or more recessive factors on chromosome II are responsible for pseudotumor phenotype.

It was next found that individuals heterozygous for ey^D (i.e., containing one pseudotumor and one non-pseudotumor fourth chromosome) showed pseudotumors. Therefore the recessive genes responsible for pseudotumor phenotype are not in chromosome four.

A study of the crossing over behavior of the pseudotumorous second chromosome was carried out subsequently. Crossing over between the genes al dp pr c and bw was found to be normal. The second

chromosome therefore does not contain any major inversions. Crosses made to flies of the tu^{49h} strain (Brncic, '49) and the mt^{A} strain (Hartung, '50) gave results which showed that the second chromosomal genes responsible for tumor production in tu^{W} are not allelic to those of the tu^{49h} or mt^{A} strain.

TABLE 14
Incidence of Normal Phenotype Among Flies of Different Genotype

	% of flies pseudotumor-free	N	I	II	III
1)	0	801	tu/Y ;	tu/tu ;	tu/tu
2)	0	805	y/Y ;	tu/tu ;	Sb/D
3)	0	246	y/Y ;	tu/tu ;	Sb/tu
4)	0	646	tu/Y ;	tu/tu ;	Sb/tu
5)	2.0	650	tu/tu ;	tu/tu ;	tu/tu
6)	3.0	325	y/tu ;	tu/tu ;	Sb/tu
7)	4.0	805	y/y ;	tu/tu ;	Sb/D
8)	4.2	451	tu/Y ;	tu/tu ;	Sb/D
9)	5.9	624	tu/tu ;	tu/tu ;	Sb/tu
10)	17.6	510	tu/Y ;	tu/tu ;	D/tu
11)	35.6	216	y/Y ;	tu/tu ;	D/tu
12)	36.0	556	tu/tu ;	tu/tu ;	Sb/D
13)	67.2	328	tu/tu ;	tu/tu ;	D/tu
14)	96.7	270	y/tu ;	tu/tu ;	D/tu
15)	99.75	786	tu/Y ;	Pm/Cy ;	tu/tu
16)	100.0	928	tu/tu ;	Pm/Cy ;	tu/tu

tu = pseudotumor chromosome
Y = Y-chromosome
y = y^{Si} sc^{8} InS y^{3P}
Pm = dp b Pm^{1}
Cy = al^{2} In(2R)Cy lt^{3} sp^{2}
D = ru h D Ins CXF ca
Sb = Sb In (3R)

See Bridges and Brehme ('44) for key to symbols used.

Crosses were next made between females of the y, Sb/D stock referred to above and Canton-S wild type males. The resulting F_1 heterozygous males or females were back crossed to y, Sb/D females or males. If F_1 males were used in the cross then no crossing over occurred between pseudotumor and C.S. second chromosomes; if F_1 females were used the reverse was true. The F_1 individuals belonged to two equal classes with respect to their second chromosomal complement: tu^{W}/tu^{W} and $tu^{W}/+$. Since only the former class would be expected to show tumors, the observed values for pseudotumor incidence were doubled, so the data (cf. Table 15) could be compared with that presented in Table 14. It is obvious that the inci-

dence of pseudotumors is lower (75% the noncrossover value on the average) in those cases where crossing over in the second chromosome was allowed to occur. It therefore appears that more than one second chromosomal gene is responsible for pseudotumor phenotype.

Next, a comparison was made (cf. Table 16) between the incidence of pseudotumors in F_2 individuals resulting from reciprocal backcrosses of C.S./tu^w heterozygotes. A difference in pseudotumor incidence between (A) and (C) males and (A) and (C) females would indicate a cytoplasmic effect. Differences between (B) and (D) males would indicate an effect dependent upon the nature of the Y chromosome. Such differences as do exist are not considered significant.

TABLE 15
Incidence of Normal Phenotype Among Flies of Different Genotype

Crossing Over Genotype				No Crossing Over Genotype			
% of flies pseudotumor-free	N	I	III	% of flies pseudotumor-free	N	I	III
16	239	y/Y	Sb/+	13	264	y/Y	Sb/+
27	169	y/Y	D/Sb	0	223	y/Y	D/Sb
38	235	y/y	Sb/+	21	400	y/y	Sb/+
39	204	+/Y	Sb/+				
47	176	+/Y	D/Sb				
52	126	y/y	D/Sb	46	228	y/y	D/Sb
56	150	y/Y	D/+	38	220	y/Y	D/+
61	118	y/y	D/+	40	243	y/y	D/+
73	255	y/+	Sb/+				
81	125	+/Y	D/+				
88	133	y/+	D/Sb				
88	118	y/+	D/+				

+ = Canton-S chromosome.

TABLE 16
Data on Pseudotumor Incidence in the F_2 of Reciprocal Backcrosses.

Cross I

P_1 C.S. female x tu^w male

F_1 female x tu^w male

(A) F_2 971 males (17.4% tu)
1079 females (11.2% tu)

F_1 male x tu^w female

(B) F_2 418 males (48.3% tu)
452 females (24.1% tu)

Cross II

P_1 C.S. male x tu^w female

F_1 female x tu^w male

(C) F_2 1875 males (16.3% tu)
1865 females (9.3% tu)

F_1 male x tu^w female

(D) F_2 656 males (44.5% tu)
668 females (26.2% tu)

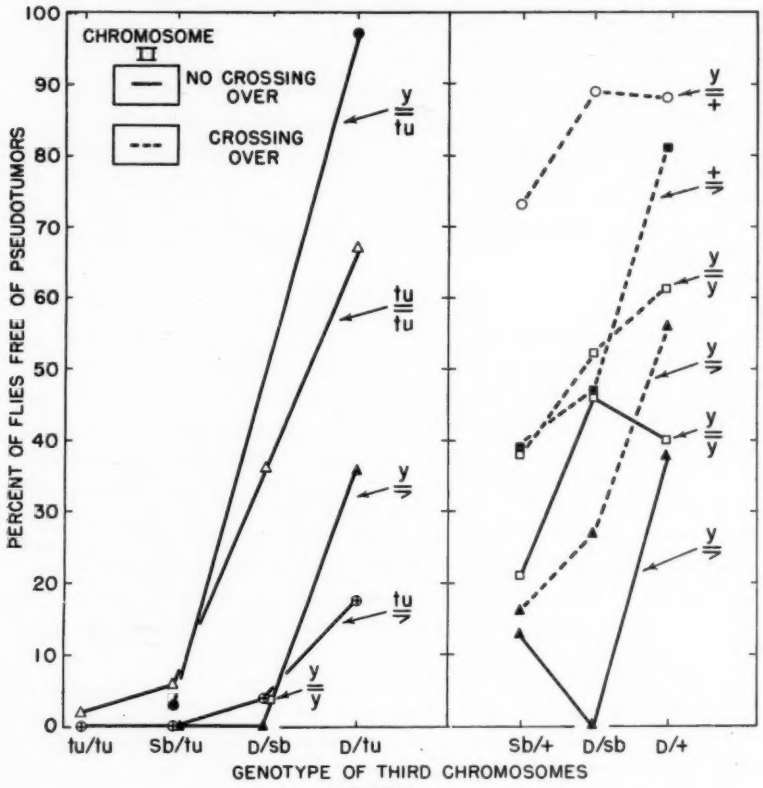
(D) females (tu^wX/tu^wX) and (B) females (tu^wX/C.S.X.) have nearly equal frequencies of pseudotumors. (A) and (C) females show a "tumor" incidence less than one half that of (B) and (D) females. The conclusion reached from the latter data is that this difference is due to lowered effectiveness of crossover products in producing pseudotumor phenotype. Previous data (cf. Table 15) showed that flies containing a product of crossing over between the pseudotumor and C.S. second chromosomes and an intact pseudotumor second chromosome showed a lower incidence of pseudotumors than flies containing two intact tu^w second chromosomes. Since the reduction in pseudotumor incidence is more striking in the present instance, it appears that crossing over in chromosomes other than the second is responsible.

Conclusions

The relation between genotype and the percent of flies free of pseudotumors is shown in Figure 7. From these data an overall description of the present genotypic basis of pseudotumor phenotype can be given. Pseudotumor phenotype is dependent upon two or more recessive genes on the second chromosome. Given two second chromosomes of pseudotumor origin the incidence of pseudotumors depends upon the number and type of the X and the type of the third chromosomes present. To explain this interaction we assume that developing flies with two pseudotumor second chromosomes produce less of a substance (tu+ substance) which is necessary at or above a certain concentration for non-tumor phenotype than do flies with two wild type second chromosomes. In this regard it is interesting to note that Lewis ('54) using filter paper chromatographic methods for the detection of free amino acids in the body fluids of mid-third instar *Drosophila* larvae found that a pseudotumor-bearing strain lacked cystine and an unidentified substance, both of which were present in a non-tumor strain.

We can speculate further that below the critical concentration of the tu+ substance the plasma constitution is so changed as to effect the surface state of certain free-floating cells. This change causes their aggregation into large clumps and makes impossible their removal by the normal reactions available to the larva. As the aggregates grow in size they tend to adhere to various organs. Melanization of the aggregate is the method adopted to make it innocuous. By

some alternative route the tu+ substance can be supplied by two or more genes on the X and on the third chromosomes. The quantity depends upon the source of these chromosomes. If one C.S. X sup-


CHROMOSOME
III
NO CROSSING OVER
CROSSING OVER
PERCENT OF FLIES FREE OF PSEUDOTUMORS
100
90
80
70
60
50
40
30
20
10
0
y/tu
tu/tu
y/y
tu/tu
y/+
+/+
y/y
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This is exactly the observed situation with the exception of $tu^w/+$ females which have a pseudotumor incidence equal to tu^w/tu^w females. Therefore an interaction between the C.S. and tu^w X chromosomes yielding a reduction in the amount of tu+ substance must be postulated. With respect to the tu^w X chromosomes, the C.S. X and the y^{S1} sc^8 InS y^{3P} X would be said to contain stronger "suppressor genes".

To explain the interactions of the third chromosomes one must assume that genes in the ru h D Ins CXF ca chromosome initiate the production of more of the tu+ substance than is the case for the Sb In (3R) chromosome, which in turn produces more than the tu^w or C.S. third chromosomes (which are more or less equal). A non-additive interaction between D and Sb chromosomes must be postulated which yields a lowered production of tu+ substance. Such a hypothesis would predict that flies placed in the order of highest pseudotumor incidence should fall into the following series: $tu^w/tu^w > Sb/tu^w = Sb/+ > D/Sb > D/tu^w = D/+$. This is the pattern which is generally observed.

The development of such a complex genetic situation may be explained if it is assumed that when the mutant was originally detected it was the result of a relatively few recessive second chromosomal mutations. Constant artificial selection for "tumor" phenotype during approximately 400 generations led to the accumulation of X, II and III chromosomal modifiers which enhanced "tumor" incidence. Another way of stating the situation is that the hypothesized tu+ substance is kept at normal concentration in the wild type strain by contributions of different size from a number of sources. Among the early mutations was one on chromosome II which cut off a major source of this substance and thus reduced the overall concentration. Through selection for "tumor" expression other mutants which cut off other sources of the tu+ substance were accumulated. Eventually the concentration was so lowered that pseudotumor incidence reached 100%. Possibly the other characteristics of the pseudotumor strain have as their origin the lowered concentration of the tu+ substance.

The above data can be explained by numerous alternative hypotheses. Another is that the tu+ substance is responsible for inhibiting the melanization process which makes the cell aggregates detectable in adults. Thus, if injection of an extract from wild type larvae

was shown to lower the incidence of pseudotumors in the tu^w hosts, one would have to show that non-melanized aggregates were not present.

Burton ('55) demonstrated that a cellular larval extract from another strain (tu-e) characterized by melanotic masses when injected into the larvae of a non-tumor strain induce "tumors" in the hosts, and therefore he concluded that an extractable carcinogen is present in this strain of *Drosophila melanogaster*. Furthermore, a complex pattern of "tumor" induction has been shown to exist which varies with the age of the host and the donor. We feel that such a conclusion is only one of many interpretations, and its validity has not been conclusively proven. Injection of haemolymph of changed plasma composition resulting from the absence of some normally occurring component might initiate cellular aggregation in the host. The physical and chemical constitution of the haemolymph and the concentration and behavior of the various types of cells within it undoubtedly varies during normal larval development (cf. Dennell, '47, '49 and Rizki, '53). One would expect an even greater variation in the haemolymph of larvae of pseudotumorous strains because internally buffering mechanisms characteristic of the wild type condition may be disturbed. The interaction between host and the injected extract might vary in the observed fashion, if the cellular composition of host haemolymph and the plasma constitution of the donor varied in the appropriate way during larval development. It is hoped that this controversy will be cleared up as more data become available.

SUMMARY

A strain of *Drosophila melanogaster* was studied which is characterized by lowered yields of larvae from eggs laid, lowered adult longevity, lowered resistance of adult flies to starvation and desiccation, an abnormal pattern of larval growth, the production of smaller eggs, failure by females to lay a large fraction of formed eggs, and a high incidence of pseudotumors. The inheritance of the pseudotumor phenotype was shown to be a complex situation due to recessive genes on chromosomes X, II and III. The major factor appears to be on chromosome II. A hypothesis is advanced to explain the origin of this complex genetic system.

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BOOK REVIEW

A Methodological, Psychiatric and Statistical Study of a Large Swedish Rural Population. Tage Larsson and Torsten Sjögren. (Tr. by Donald Burton) Acta Psychiatrica et Neurologica Scandinavica Supplementum 89. Ejnar Munksgaard, Copenhagen, 1954. 254 pages.

The aim of the study was to make a statistical and genetic analysis of psychoses and low-grade oligophrenia over a relatively long period of time and in a geographically limited area. The investigators are from the Karolinska Institutet of the University of Stockholm.

Sweden offers very favorable possibilities for population studies and family investigations. Careful statistics of the population have been kept and cases of mental disease and mental deficiency must, by law, be recorded in the parish registers. The investigation covers forty-five years of registration for a rural area with about 25,000 inhabitants. The area included in the investigations consist of 13 parishes together comprising two large islands and a number of adjacent smaller islands off the west coast of Sweden. This setting was particularly appropriate for the investigation since the annual influx of new residents to the district is very low. Although the frequency of removals from the district is high, the great majority of these are to the nearby city of Gothenburg and two other districts in the same county. The investigation covers the period January 1, 1900 through December 31, 1944.

The basic material for the study was obtained by going through parish registers for the period of the study. Other sources and cross checks included the hospital records and the register of welfare organizations for the same period. Materials from the three sources were coordinated. The records were collected in such a way as to permit analysis of the population resident within the area as well as calculations for those born in the area. Various precautions were undertaken to avoid the problem of over estimation and under estimation resulting from movement and from time lag in incidence.

The probands comprise all the persons registered in the three basic sources who had not died before January 1, 1900 and who met the specifications of the diagnosis. They consist of 1312 registered cases of psychosis, severe psychopathy, low-grade oligophrenia, and suicide.

The search in the parish registers and field investigations yielded very complete genealogical information concerning the families of the probands and secondary cases born on the islands.

The detailed results and relationships are described in 56 tables. Only the most generalized results can be reported here. The following figures will be of some interest to students of population, genetics, and the care of the retarded and the disturbed.

FINAL ASSESSMENT OF MORBIDITY-RISKS
(Aggregate to 80 years)

Category	*Men* %	*Women* %
Schizophrenia	1.6	1.6
Manic-depressive psychosis	0.9	1.2
Senile and pre-senile psychosis	1.3	1.8
Other psychosis	0.9	1.1
All psychoses	4.7	5.7
Low-grade oligophrenia (over 10 years)	1.0	0.75

A concluding chapter predicts the numbers for 1965 as compared to 1945. The second report will be concerned with familial incidence and relationships.

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